CHARACTERIZATION OF SOME CARBOSTYRIL CONGENERS HAVING NOVEL BETA-ADRENORECEPTOR AGONIST PROPERTIES

By

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A DISSERTATION SUBMITTED TO THE GRADUATE SCHOOL
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This dissertation is dedicated to my parents and grandparents whom I love very much. They have taught me that even the most outrageous goals can be more than just dreams.

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The interaction of two new derivatives of 8-hydroxy-carbostyril with the beta-adrenoreceptor system was partially characterized using isolated membranes, cultured cells, and animals. The carbostyril congeners contained an amino (Carbo-Am) or bromoacetamido (Carbo-Br) moiety in the para position of the phenyl ring.

In rat reticulocyte membranes, binding studies under different conditions with [^{125}I]iodocyanopindolol revealed that Carbo-Br and Carbo-Am were 17 to 107-fold more potent than (-)isoproterenol. Carbo-Am and Carbo-Br were 13.5 and 30-fold, respectively, more potent than (-)isoproterenol at stimulating adenylate cyclase activity, but exhibited the same intrinsic activity. Adenylate cyclase activation by Carbo-Br, Carbo-Am or (-)isoproterenol was blocked with concurrent addition of propranolol. If, however,

propranolol was added 7 min into the assay, further cAMP production by Carbo-Br and Carbo-Am was unaffected while further cAMP production by (-)isoproterenol was immediately blocked. Both Carbo-Br and Carbo-Am induced a receptor loss that was blocked by nadolol. The tightly bound Carbo-Am could be partially dissociated by heat treatment whereas Carbo-Br could not.

Using DDT_1-MF-2 cells, Carbo-Br produced a desensitization much like (-)isoproterenol, yet appeared to be binding irreversibly to the beta-adrenoreceptor.

In vivo, Carbo-Br stimulated rat heart and lung ornithine decarboxylase activity. This stimulation was blocked by propranolol. Three hours after an injection of Carbo-Br (0.5-10.0 mg/kg), there was a dose-dependent loss of up to 75% of beta-adrenoreceptors in lung and spleen. Maximal loss of binding in heart and submaxillary gland was only 25%. In the four tissues tested, the $beta_2$-receptor population showed the greatest loss of specific binding.

These results indicated that the two carbostyril congeners were potent, full, beta-adrenoreceptor agonists that produced sustaineded activation effects in membranes and bound receptors tightly and/or irreversibly. Carbo-Br produced desensitization in intact cells and appeared to show irreversible selectivity for the $beta_2$ subtype. Both congeners could be useful tools with which to further probe beta-adrenoreceptor turnover, processing, and regulation.

CHAPTER 1
INTRODUCTION

Function

The catecholamines, epinephrine (Epi) and norepinephrine (NE), have been shown to be responsible for a wide spectrum of biochemical, physiological, and pharmacological effects. Ahlquist (1948) clarified the previous confusion concerning the actions of norepinephrine, epinephrine, and isoproterenol causing excitation or inhibition of smooth muscle contraction, depending on the site, dose and catecholamine given. He found norepinephrine to be the most potent excitatory catecholamine, with very low activity as an inhibitor. Isoproterenol (Iso) exhibited the reverse actions. Epinephrine was found to be relatively potent in both actions. On the basis of these observations, Ahlquist proposed the terms alpha- and beta-receptors for the sites on smooth muscle where catecholamines produced these responses. He distinguished the two receptor sites by the following potency series: alpha = Epi > NE >> Iso, and beta = Iso > Epi > NE. These distinctions were later supported by the finding that there are antagonistic drugs which are specific for each receptor as well (alpha: phentolamine, phenoxybenzamine; beta: propranolol, dichloroisoproterenol). In 1967, Lands *et al.*(a,b) proposed dividing beta-adrenoreceptors into two

subtypes, $beta_1$ and $beta_2$, based on the relative selectivity of both excitatory agents and antagonists. $Beta_1$-adrenoreceptors, found primarily in the heart and adipose tissue, showed the potency series Iso > Epi = NE. $Beta_2$-adrenoreceptors were found in the bronchi and vascular smooth muscle and showed the series Iso > Epi > NE. Though selectivity has been seen only at low doses, further support for this classification has come from observations that antagonists practolol, metaprolol and atenolol were more effective at blocking responses in heart, whereas certain agonists (salbutamol and terbutaline) were more selective for $beta_2$-receptors. Both $beta_1$- and $beta_2$-adrenoreceptors were shown by Sutherland and coworkers (1965) to be coupled to the membrane bound enzyme adenylate cyclase. Occupancy of the receptor by an agonist stimulated the enzyme which increased the intracellular concentration of adenosine 3'5'-cyclic monophosphate (cAMP). The beta-adrenoreceptor regulated cyclase system was composed of three separate components: receptor (R), stimulatory guanine nucleotide regulatory protein (N_S), and the adenylate cyclase catalytic moiety (De Lean *et al.*, 1980; Limbird *et al.*, 1980a; Levitzki, 1978; Rodbell, 1980). Interaction of an agonist (H) with the receptor has been shown to result in the initial formation of a low affinity, freely reversible complex, H-R (Heidenreich *et al.*, 1980; Kent *et al.*, 1980; Williams & Lefkowitz, 1977; Lefkowitz *et al.*, 1978). A conformational change was then induced in the receptor such that the receptor became coupled

to the N_S-protein (Citri & Schramm, 1980; Limbird *et al.*, 1980a). The ternary complex, or H-R-N_S, has been shown to include high affinity agonist binding which was slowly dissociable (Heidenreich *et al.*, 1980; Kent *et al.*, 1980; Williams & Lefkowitz, 1977; Wessels *et al.*, 1978). Formation of this ternary complex has been shown to be a prerequisite for activation of cyclase (De Lean *et al.*, 1980; Limbird *et al.*, 1980a). In forming this complex there was a loss of tightly bound guanosine 5'-diphosphate (GDP) from N_S, and a subsequent facilitation of binding of guanosine 5'-triphosphate (GTP) to the N_S-site (Cassel & Selinger, 1978). Interaction with GTP destabilized the complex, freeing H-R and releasing free N_S-GTP. Free N_S-GTP then interacted with the inactive enzyme and activated it (Pfeuffer, 1979). Cleavage of GTP to GDP by GTPase located on N_S returned the cycle to its basal state (Cassel & Selinger, 1976; 1977).

Regulation

The term desensitization has been defined as a "phenomenon in which the response of a tissue or cell to a biologically active agent becomes attenuated with time in the presence of a continuing stimulus of constant intensity" (Sibley & Lefkowitz, 1985). Desensitization has been commonly observed in the beta-adrenoreceptor-coupled adenylate cyclase system.

Two types of desentization have been described (Stiles *et al.*, 1984; Harden, 1983). The first, homologous (or agonist-specific) desensitization, occurred when an

attenuated responsiveness was observed only to the desensitizing agonist, with no loss of responsiveness to any other agonist. Conversely, a heterologous desensitization was observed when exposure of one agonist to a cell decreased the response to multiple agonists operating through distinct receptors (such as prostaglandin E_1, PGE_1, and glucagon).

In both cases, the beta-adrenoreceptor has been shown to be phosporylated during the desensitization process. Recent evidence linked this covalent modification to the decreased functionality of the receptor (Benovic *et al.*, 1985; Sibley *et al.*, 1984; Strulovici *et al.*, 1984; Stadel *et al.*, 1983). These studies suggested that phosphorylation of the receptor was involved in regulating the hormonal responsiveness of the adenylate cyclase system. Several patterns of phosphorylation of these receptors by various kinases have been characterized with respect to their dependence on agonist occupancy.

Phosphorylation of the $beta_2$-adrenoreceptor by protein kinase C has been shown to be an agonist-<u>independent</u> process (Bouvier *et al.*, 1987; Fishman *et al.*, 1987), whereas the rate of phosphorylation of $beta_2$-adrenoreceptors by cAMP-dependent protein kinase was increased by agonist occupancy of the receptors (Bouvier *et al.*, 1987; Yamashita *et al.*, 1987; Benovic *et al.*, 1985). In the latter case, however, the cAMP-dependent protein kinase was responsible for only one-half of the desensitzation, the rest attributed to other kinases. Since many different drugs or hormones have been

shown to activate cAMP-dependent protein kinase and protein kinase C *in vivo*, these types of phosphorylation reactions were good candidates for the development of heterologous desensitization.

Identification of a new cAMP-independent protein kinase, beta-adrenergic receptor kinase (BARK) (Benovic *et al.*, 1986), which preferentially phosphorylated the agonist-occupied form of the receptor, suggested a mechanism for homologous desensitization. The enzyme phosphorylated the receptor on several serine and threonine residues near the carboxyl terminus of the receptor (Benovic *et al.*, 1986).

Purification of BARK (Benovic *et al.*, 1987a) yielded a cytosolic protein with molecular weight (M_r) of 80,000. Agonist occupancy of the beta-adrenoreceptor caused a translocation of BARK from the cytosol to the plasma membrane, and this translocation was concurrent with the time courses of receptor phosphorylation, desensitization, and sequestration (Strasser *et al.*, 1986). Agonist binding to another stimulatory adenylate-cyclase coupled receptor, PGE_1-receptor, and to an inhibitory adenylate cyclase-coupled receptor, $alpha_2$-adrenoreceptor (Benovic *et al.*, 1987b), also promoted translocation of BARK, suggesting that BARK may have served to phosphorylate and desensitize other adenylate cyclase-coupled receptors as well. However, only agonist occupied receptors were substrates for the kinase, consistent with homologous desensitization.

The mechanisms by which receptor phosphorylation led to desensitization have remained unclear. The possibility that the phosphorylated receptor has diminished ability to interact with the guanine nucleotide regulatory protein was a mechanism that was consistent with the actions of cAMP-dependent protein kinase in heterologous desensitization. Studies on human astrocytoma cells (Su *et al.*, 1980; Harden *et al.*, 1979) demonstrated that desensitization preceded receptor sequestration, an event characterized by a loss of binding of cell surface receptors. It has been shown that receptor phosphorylation triggers the sequestration event (Benovic *et al.*, 1986). Only agonist-occupied receptor phosphorylation, and not cAMP-induced receptor phosphorylation, has resulted in receptor sequestration or internalization (Sibley *et al.*, 1987 and Toews *et al.*, 1987).. An alternative mechanism (Clark *et al.*, 1988) suggested that phosphorylation was only a signal that resulted in desensitization; i.e., desensitization did not occur until the physical isolation of the receptor from the N_S-protein was complete.

Structure

The beta-adrenergic receptors have been shown to be integral membrane glycoproteins of M_r = 64,000 (Kobilka *et al.*, 1987a). The complete primary amino acid sequences of the mammalian $beta_1$- (Frielle *et al.*, 1987) and $beta_2$-adrenoreceptors (Dixon *et al.*, 1987a; Kobilka *et al.*, 1987a) were recently deduced. Though the amino acid sequence was highly

conserved, distinct genes code for each subtype (Emorine *et al.*, 1987). Each protein has been shown to consist of 418 amino acids which form seven membrane spanning regions. The striking sequence homology between the beta-adrenergic, alpha-adrenergic, muscarinic cholinergic, and opsin families of receptors have led many to theorize that a large "Super-gene" or multi-gene family of receptors exists (Gocayne *et al.*, 1987; Lefkowitz & Caron, 1988). On the basis of the homology between the amino acid sequence of the beta-adreno-receptors and the opsin proteins, Dixon *et al.* (1987b) proposed that the ligand binding domain must lie within the seven hydrophobic domains. By producing genes for hamster beta$_2$-adrenoreceptor with sequential deletions, the same group showed that the transmembrane regions were required for the structural integrity of the receptor, and that two cysteine residues in the receptor were important for agonist binding affinity. A deletion of eight amino acids in the fourth membrane spanning region resulted in the ability of the agonist to bind with high affinity but <u>not</u> to stimulate adenylate cyclase activity (Dixon *et al.*, 1987a). Substi-tution on amino acid 79 in the second membrane spanning region resulted in a receptor that displayed normal binding for antagonists, but reduced affinity for agonists (Strader *et al.*, 1987b; Chung *et al.*, 1988).

Site-directed mutagenesis was also used to study the importance of specific receptor regions in desensitization (Strader *et al.*, 1987a; Kobilka *et al.*, 1987b; Fraser *et al.*,

1987). Mutations were introduced that resulted in deletions of the carboxyl terminus, putative cAMP-dependent protein kinase substrate sites, and the N_S-protein coupling site. Only the mutation which perturbed coupling of the receptor and N_S-protein caused an altered sequestration response. This suggested a correlation between coupling of the receptor to cyclase and the ability of the receptor to undergo agonist-mediated sequestration.

As briefly reviewed above, much has been established about the subtypes of the beta-adrenoreceptor, the mechanism of receptor mediated cyclase activation, receptor regulation, and desensitization. In addition, the recent cloning of the receptor has led to a major focus on structural studies of the receptor. Even with these advances, a great deal has remained in question: the precise interaction of the receptor with the stimulatory guanine nucleotide binding protein, a detailed examination of the relationship between receptor number and the ability of the receptor to produce a response, a description of the basal turnover of the receptor, the cellular components involved in receptor processing, and the processing of the receptor after agonist-induced internalization. These questions could only be answered by studying the receptors in their homeostatic environment. In the past, a number of irreversible antagonists have been synthesized to attempt to study some of these questions (Pitha *et al.*, 1980; Dickinson *et al.*, 1985; Kusiak & Pitha, 1987). However important differences between

agonist and antagonist binding have been shown to exist. Agonist binding showed several affinity states (Stiles *et al.*, 1984), and was modulated by guanine nucleotides (Lefkowitz *et al.*, 1976; Maguire *et al.*, 1976), divalent cations (Bird & Maguire, 1978; Williams *et al.*, 1978), temperature (Scarpace *et al.*, 1986; Baker & Potter, 1981; Briggs & Lefkowitz, 1980; Weiland *et al.*, 1979; Insel & Sanda, 1979), and N-ethylmaleimide (Vauquelin *et al.*, 1980; Vauquelin & Maguire, 1980). In contrast, antagonist binding usually showed only a single affinity state and was not affected by those modulators (Heidenreich *et al.*, 1980; Lefkowitz *et al.*, 1978). In light of the differences between agonist and antagonist binding, it would be useful to characterize the effects of an agonist that binds irreversibly to the beta-adrenoreceptor. Another important question has been whether an irreversibly bound agonist could produce a sustained beta-adrenergic response in a multicomponent receptor system. Towards this end, a program was initiated to design and synthesize some potent and stable beta-adrenergic ligands based on a carbostyril nucleus (Milecki *et al.*, 1987), the structure of two of which can be seen in Figure 1-1. The goal of this work has been to examine beta-adreno-receptor mediated properties of these ligands with the implicit hypothesis that the bromoacetylated compound was an irreversible agonist.

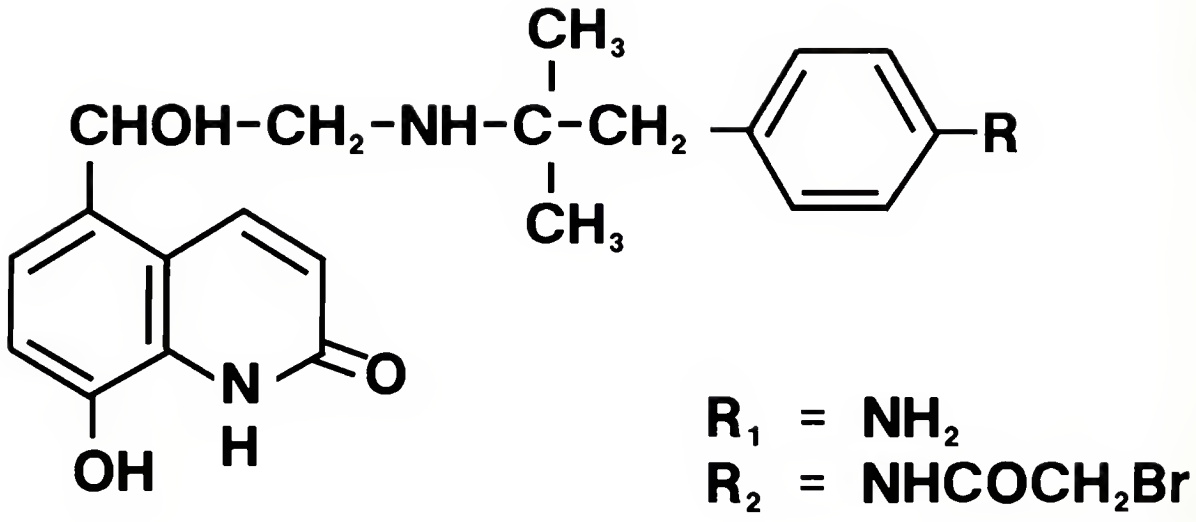

Figure 1-1. Structures of Carbostyril compounds Carbo-Am (R_1) and Carbo-Br (R_2). The 8-hydroxycarbostyril moiety is the double-ringed structure on the lower left.

CHAPTER 2
MEMBRANE STUDIES

Introduction

Beta-adrenergic activation of adenylate cyclase activity has been shown to involve a complex set of interactions of several components in the plasma membrane. These components included at least the beta-adrenoreceptor, a stimulatory guanine nucleotide binding protein (N_S) and the catalytic unit of adenylate cyclase (Levitzki, 1978; Rodbell, 1980). One useful approach in the study of the beta-adrenoreceptor has been the development of several irreversible antagonists. Irreversible receptor antagonists have been used to study the relationship between receptor number and biological responses (Homburger *et al.*, 1985; Posner *et al.*, 1984; Venter, 1979; Tolkovsky & Levitzki, 1978), receptor recovery after irreversible blockade in cultured cells (Hughes & Insel, 1986; George *et al.*, 1986; Homburger *et al.*, 1984; Fraser & Venter, 1980) or whole animals (Baker *et al.*, 1986; Nelson *et al.*, 1986; Baker & Pitha, 1982), and the antagonist binding subunit of the receptor (Minneman & Mowry, 1986; Stiles *et al.*, 1984; Atlas & Levitzki, 1978). To date, most agonist-receptor interaction studies have been performed indirectly by competing unlabelled agonists with labelled antagonists.

Although some studies have used labelled agonists, these compounds were generally unstable, had low specific activities, and showed high nonspecific binding (Giudicelli *et al.*, 1982; Heidenreich *et al.*, 1980; Lefkowitz & Williams, 1977). An irreversible agonist would have been helpful in characterizing the differences between agonist and antagonist interaction with the beta-adrenoreceptor and the complex interactions of the components in that system. Towards that end, an alkylating derivative of norepinephrine was recently reported to be a partial beta-adrenergic agonist which could irreversibly bind to the beta-adrenoreceptor (Baker *et al.*, 1985). However, though this compound initially stimulated adenylate cyclase activity, it appeared to act as an antagonist after receptor alkylation and was, like most catecholamines, unstable. Recently we reported on the synthesis of several stable carbostyril derivatives which preliminary evidence indicated were potent beta-agonists (Milecki *et al.*, 1987). The synthesis of this type of compound was based on an earlier report that carbostyril-based compounds were stable beta-agonists (Yoshizaki *et al.*, 1976). Figure 1-1 showed two of the carbostyril compounds used in the present study. A more detailed examination was made on the interaction of these two derivatives with the beta-adrenoreceptor system of rat reticulocytes. Evidence has been provided to indicate that, as hypothesized in the first chapter, they produce an extremely tight and/or

irreversible activation of adenylate cyclase activity *in vitro*.

Experimental Procedures

Source of Materials

The radioligands (-)-[^{125}I]Iodocyanopindolol ([^{125}I]CYP; 2000-2200 Ci/mmol) and [2,8-^{3}H]adenosine 3'5'-cyclic monophosphate ([^{3}H]cAMP; 31.2 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL, USA) and New England Nuclear (Boston, MA, USA), respectively. ATP, guanyl-5'-yl-imididodiphosphate (Gpp(NH)p), GTP, protein kinase, (±)alprenolol, hydroxyapatite, Iso, theophylline, creatine phosphokinase, phosphocreatine, penicillin G, streptomycin sulfate, and amphotericin B were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nadolol was a gift from Dr. A. L. Bassett, University of Miami (Miami, FL, USA), and propranolol was a gift from Ayerst Laboratories Inc. (New York, NY, USA). The GH_3 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Media, fetal bovine serum and horse serum were purchased from Gibco (Grand Island, NY, USA). Liquiscint was purchased from National Diagnostics (Somerville, NJ, USA).

Methods

Drug Preparations. The synthesis and chemical characterization of 5-[2-[[1-(4-aminophenyl)-2-methylprop-2-yl]amino]-1-hydroxyethyl]-8-hydroxycarbostyril (Carbo-Am) and

5-[2-[[3-[4-(bromoacetamido)phenyl]-2-methylprop-2-yl]amino]-1-hydroxyethyl]-8-hydroxycarbostyril (Carbo-Br) has been previously described in detail (Milecki *et al.* 1987), and was received from this group. Stock solutions (1 mM) were made with ethanol and diluted with either water or buffer to give the desired concentrations.

In some experiments, the Carbo-Br compound (0.5 mM) was incubated without and with 2 mM cysteine in 50 mM sodium phosphate buffer pH 7.4 for 18 hr at 25°C. At the end of the incubation, samples were run on silica-gel TLC plates with chloroform/methanol (1:1). The R_f for Carbo-Br alone was 0.34 whereas for R_f for the reaction product of Carbo-Br and cysteine was 0.05. Virtually complete conversion of the Carbo-Br occurred. The reaction product, when used, was referred to as carbo-cysteine.

Buffer. Unless otherwise noted, buffer was 50 mM Tris-HCl pH 7.4 containing 5 mM $MgCl_2$.

Rat Reticulocyte Preparation. Reticulocyte production was induced by a slight modification of a previously described technique (Baker *et al.*, 1985). Briefly, male Sprague-Dawley rats (175-225 g) were injected (subcutaneously) on days one, three, and four with 25, 30 and 35 mg/kg, respectively, of phenylhydrazine hydrochloride to induce hemolysis. The drug was prepared by dissolving in water and adjusting the pH to 7.0 with solid sodium bicarbonate. On day seven, blood was withdrawn by cardiac puncture and the red cells washed twice at 4°C in 20 mM Tris-HCl at pH

7.4 containing 150 mM NaCl and 20 mM sodium citrate. The cells were then washed (by centrifugation at 800xg for 10 min and gentle resuspension) twice more in 20 mM Tris-HCl at pH 7.4 containing 150 mM NaCl. The cells were then lysed by a 20-fold dilution in ice-cold 10 mM Tris-HCl pH 7.4 containing 2 mM $MgCl_2$ followed by homogenization in a Waring blender at top speed for 2 min. The suspension was centrifuged at 48,000xg for 10 min and the top layer of the bilayered pellet was swirled free. The top layer of membranes was then washed (by centrifugation at 48,000xg for 10 min and resuspension) twice in buffer and stored in buffer under liquid nitrogen until used. The protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Maximal specific [^{125}I]CYP binding in these membranes ranged from 0.5 to 0.8 pmol/mg protein.

Rat erythrocyte membranes were prepared by withdrawing blood by cardiac puncture, washing and lysing the red blood cells as described above for reticulocytes. After lysis, the suspension was centrifuged at 48,000xg for 10 min and the pellet resuspended in ice-cold buffer. The suspension was centrifuged again at 48,000xg for 10 min and the pellet was washed (by resuspension and centrifugation) once more with buffer. The final pellet was resuspended in 1 vol of buffer for assays and stored under liquid nitrogen.

Cell Culture. Rat pituitary tumor (GH_3) cells were grown in 57 cm^2 culture dishes with Dulbecco's Modified Eagle's Medium containing 5% horse serum, 5% fetal calf

serum, 100 U/ml penicillin G, 0.1 mg/ml streptomycin sulfate, and 0.25 mg/ml amphotericin B. The cells were grown in a 5% CO_2/95% air humidified atmosphere at 37°C. Plates were seeded at 1 x 10^5 cells/ml and the cells harvested at 80-90% confluence. The cells were washed twice on the plate with 5 mM sodium phosphate buffer at pH 7.4 containing 150 mM NaCl (PBS) and scraped free in 1 ml of PBS with a rubber policeman. The cells were then centrifuged at 1000xg for 5 min and the cell pellet resuspended in 20 vol of ice-cold buffer. The suspension was then homogenized (Tekmar SDT-100 EN, setting 3, 5 sec) and centrifuged at 48,000xg for 10 min. The pellet was then washed twice more (by resuspension and centrifugation) and the final pellet resuspended in 1 vol of buffer for assays.

Antagonist Binding Assays. Beta-adrenoreceptor content was determined by incubating membrane protein (5-10 µg) in a total volume of 0.25 ml with 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 3-100 pM [^{125}I]CYP in the presence and absence of 1 µM (±)alprenolol for 60 min at 36°C. At the end of the incubation, each suspension was diluted with 3 ml of 50 mM Tris-HCl at pH 7.4 (36°C) and poured onto a Whatman GF/B glass fiber filter under reduced pressure. The filter was washed with a further 6 ml of buffer, placed in a vial and radioactivity determined. Specific [^{125}I]CYP binding to the beta-adrenoreceptor was calculated as the difference between the total binding in the absence of (±)alprenolol and the nonspecific binding determined in the presence of 1 µM

(±)alprenolol. Nonspecific binding was the same if the (±)alprenolol was replaced with 100 μM Iso and specific binding was 90-95% of the total bound.

In some experiments, the ability of the carbostyril congeners or Iso to inhibit specific [^{125}I]CYP binding was determined. Assays were the same as above except the [^{125}I]CYP concentration was 30 pM and 0.1% sodium ascorbate was included when Iso was used. The competitive binding assays were also performed in the presence and absence of 100 μM Gpp(NH)p, a non-hydrolyzable analog of GTP. All binding assays were performed in triplicate, the results varying by less than 5%.

Adenylate Cyclase assays. Enzyme activity was determined by a slight modification of a competitive protein binding assay described previously (Baker *et al.*, 1985). Membrane protein (20-40 μg) was incubated in a total volume of 0.15 ml with 1.6 mM ATP, 5 mM $MgCl_2$, 1.0 mM ethylene glycol bis(beta-aminoethylether-N,N,N'N'-tetraacetic acid (EGTA), 10 mM theophylline, 0.1% bovine serum albumin, 50 mM Tris-HCl at pH 7.4, creatine phosphokinase (67 units/ml) and phosphocreatine (2.5 mM) for 10 min at 32°C. These were the basal conditions. When stimulation of enzyme activity was measured, the binding assay also contained 500 μM GTP, in the presence or absence of Iso or the carbostyril congeners. At the end of the incubation, 0.3 ml of 10 mM Tris-HCl buffer pH 7.0 containing 5 mM ethylenediamine tetraacetic acid (EDTA) was added to each tube and the tubes placed in a boiling

water bath for 5 min. After cooling to room temperature, the tubes were centrifuged for 5 min at 1,200xg.

The cAMP content of the supernatant was determined by incubation in a total volume of 0.2 ml containing 25 mM Tris-HCl buffer pH 7.0, 10 mM theophylline, 0.8 pmol of [^{3}H]cAMP, an aliquot of the supernatant and 8 µg of bovine heart cAMP dependent protein kinase for 60 min at 4°C. At the end of the incubation, 70 µl of a 50% (w/v) hydroxyapatite suspension was added to each tube followed by 4 ml of ice-cold 10 mM Tris-HCl buffer at pH 7.0. The suspension was then poured onto a Whatman GF/C glass fiber filter under reduced pressure. The filter was washed with a further 8 ml of ice-cold 10 mM Tris-HCl pH 7.0, and placed in a scintillation vial with 1 ml of 0.3 N HCl. After the hydroxyapatite had dissolved (about 10-15 min), 9 ml of Liquiscint was added and the radioactivity determined. The amount of cAMP present was calculated from a standard curve determined with unlabelled cAMP. The production of cAMP was linear with time and protein through 14 min and 0.1 mg, respectively.

Membrane Pretreatments. Membrane protein (2-3 mg/ml) was suspended in 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$ and other additions as indicated in the text. The suspensions were then incubated for varying times at 30°C. At the end of the incubation, the suspensions were diluted with 20 ml of ice-cold incubation buffer containing 5 mM $MgCl_2$ and centrifuged at 48,000xg for 10 min. The pellet was resuspended in 20 ml of ice-cold incubation buffer and

centrifuged again at 48,000xg for 10 min. The pellet was washed twice more by centrifugation and resuspension and the final pellets were resuspended in 1-2 ml of buffer for assays.

In heat treatment experiments, membrane protein (2-3 mg/ml) was incubated in 50 mM sodium phosphate buffer pH 7.4 containing 5 mM $MgCl_2$ and other additions as indicated in the text at 45°C for 30 min. At the end of the incubation, the suspensions were cooled in an ice-water bath and centrifuged at 48,000xg for 10 min. The pellets were then washed (by centrifugation and resuspension) three more times with buffer and the final pellets were resuspended in 2 ml of buffer for assays.

Data Analysis. The receptor concentrations and K_D values were determined from regression analysis of Scatchard (1949) plots. The concentrations of compounds which inhibited ligand binding by 50% (IC_{50}) or the effective concentrations of drugs which gave 50% of a maximal response (EC_{50}) were determined from a dose-effect analysis using an Apple IIe microcomputer as described by Chou and Talalay (1983). Statistical analysis of the data was performed using the Student's t-test.

Results

Effects of carbostyril derivatives on reticulocyte beta-adrenoreceptors. The structures of Carbo-Am and Carbo-Br were shown in Figure 1-1. Both compounds were racemic mixtures. Figure 2-1 showed the ability of Iso and the two

carbostyril derivatives to inhibit specific [^{125}I]CYP binding to rat reticulocyte membranes. In the absence of Gpp(NH)p, the concentration of Iso that inhibited [^{125}I]CYP binding by 50% (IC_{50}) was 49 ± 3 nM and the Hill slope was 0.51 ± 0.03. In the presence of 100 μM Gpp(NH)p, the IC_{50} value increased 16.5-fold to 813 ± 66 nM and the Hill slope increased to 0.94 ± 0.02 (Figure 2-1A). Figure 2-1A also showed the competition curves for Carbo-Am. In the absence of Gpp(NH)p, the IC_{50} value was 5.9 ± 0.2 nM whereas in the presence of Gpp(NH)p the IC_{50} value was 21 ± 0.6 nM. The ability of Carbo-Br to inhibit [^{125}I]CYP binding was shown in Figure 2-1B. In the absence of Gpp(NH)p, the IC_{50} value was 3.3 ± 0.3 nM and the addition of Gpp(NH)p only shifted the IC_{50} value 2.2-fold to 7.6 ± 0.3 nM.

Figure 2-2 showed a representative Scatchard plot of [^{125}I]CYP binding after pretreatment of reticulocyte membranes with Carbo-Br or Carbo-Am. As Iso is easily washed from membranes, no control for Iso pretreatment was necessary. Membranes incubated with 25 nM Carbo-Br for 30 min at 32°C followed by four washes had an 82% reduction of specific [^{125}I]CYP binding sites with no change in the K_D value of [^{125}I]CYP for the remaining receptors (control, 14 pM; Carbo-Br-treated, 12 pM). When 100 μM Gpp(NH)p was added during the pretreatment period, the loss of specific binding sites was reduced to 65% with no alteration of the K_D value (13 pM) as compared to the control for the remaining receptors (Figure 2-2A). Figure 2-2B showed a Scatchard plot after

Figure 2-1. Inhibition of specific [^{125}I]CYP binding in rat reticulocyte membranes by Iso, Carbo-Am (A) and Carbo-Br (B). Membranes were incubated with buffer at pH 7.4, 30 pM [^{125}I]CYP, the indicated concentrations of (A) Iso (squares), Carbo-Am (circles) or (B) Carbo-Br (circles) and without (open symbols) or with (closed symbols) 100 μM Gpp(NH)p for 45 min at 36°C. In the Iso competition assays, 0.1% ascorbate was also present. At the end of the incubation, the specific binding was determined as described in the "Methods" section. Each point on the graph is the mean of three determinations assayed in triplicate. Control [^{125}I]CYP binding ranged from 524 to 583 fmol/mg protein.

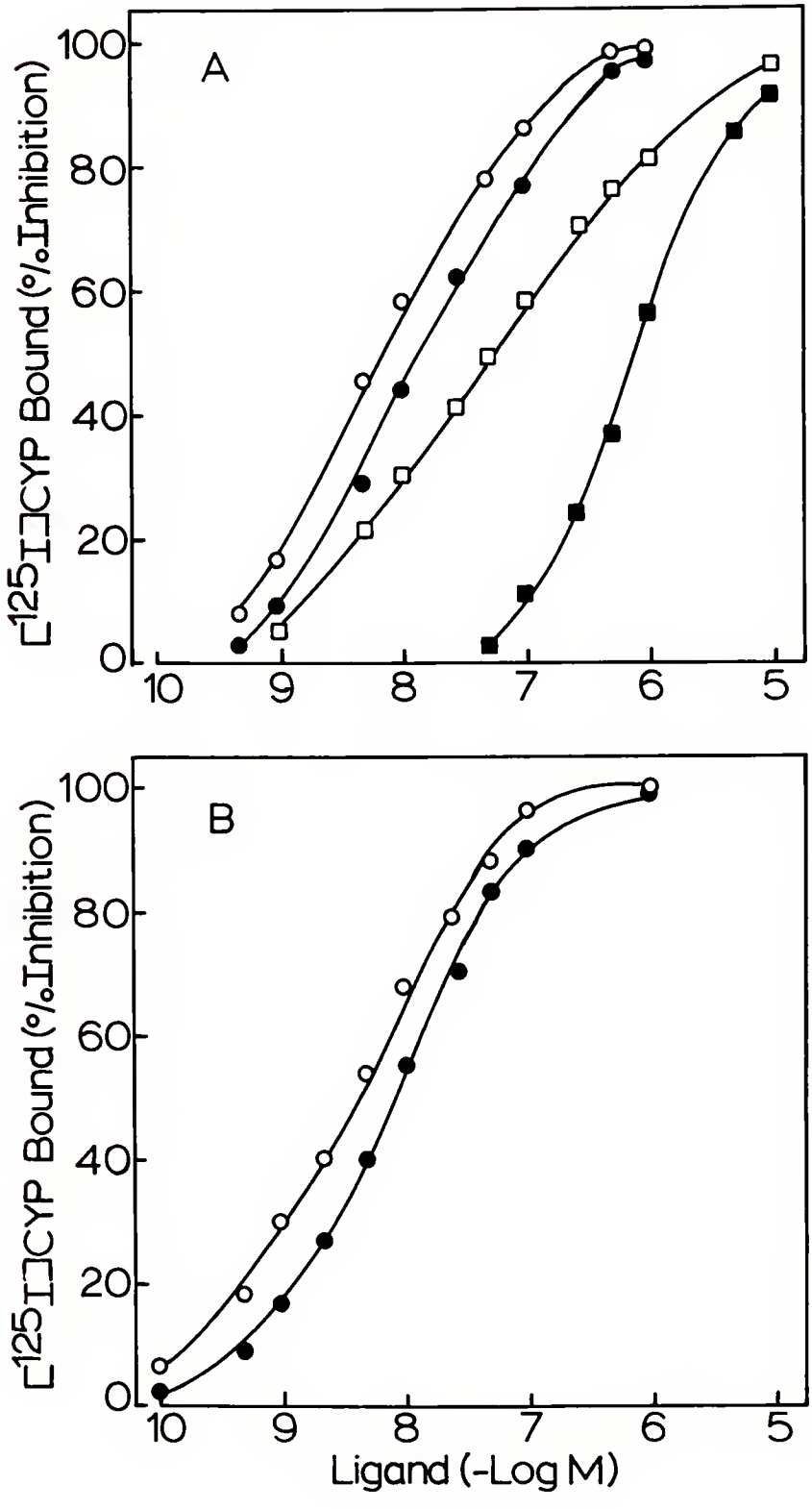
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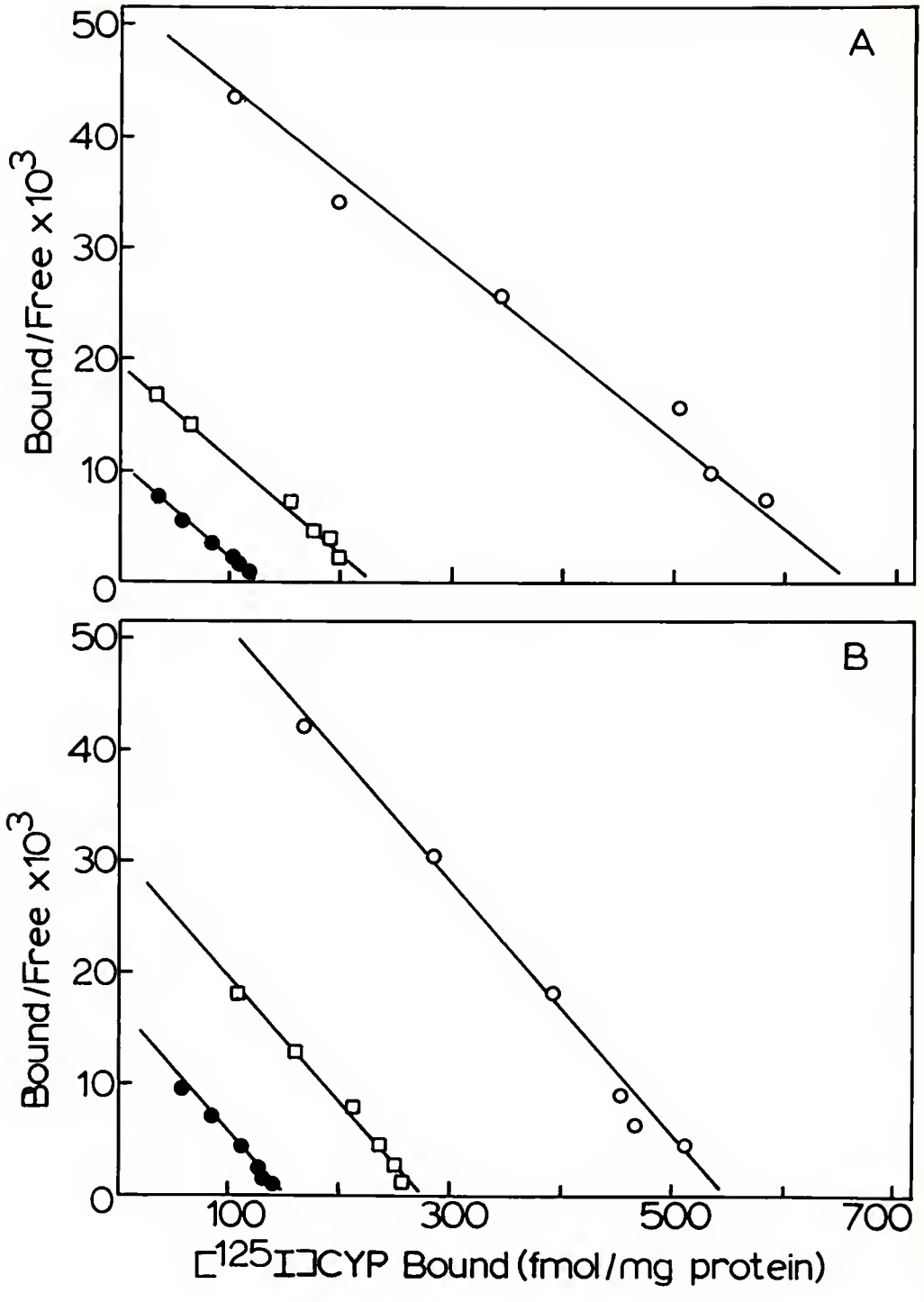
Figure 2-2. Scatchard plot of specific [^{125}I]CYP binding to rat reticulocyte membranes after treatment with (A) Carbo-Br or (B) Carbo-Am. In A, membranes were incubated in buffer without (open circles) and with (closed circles) 25 nM Carbo-Br or with 25 nM Carbo-Br plus 100 µM Gpp(NH)p (squares) for 30 min at 32°C. In B, membranes were incubated without (open circles) and with (closed circles) 0.1 µM Carbo-Am or with 0.1 µM Carbo-Am plus 100 µM Gpp(NH)p (squares) for 30 min at 32 °C. At the end of the incubation, the membranes were washed four times with buffer and assayed with 3 to 100 pM [^{125}I]CYP as described in the "Methods" section. The data were plotted as the ratio of the amount of specifically bound ligand (pmol/mg protein) to free ligand (pmol/l) versus the amount of specifically bound ligand/mg protein. Data points were the mean of triplicate determinations and are representative of three experiments.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40
30
20
10
0
100
300
500
700
[125I]CYP Bound (fmol/mg protein)

pretreatment of reticulocyte membranes with 0.1 μM Carbo-Am for 30 min at 32°C followed by four wash cycles. The Carbo-Am-treated membranes showed a 73% reduction in specific [^{125}I]CYP binding with no change in the K_D value of [^{125}I]CYP for the remaining receptors (control, 8 pM; Carbo-Am-treated,10.4 pM). Inclusion of 100 μM Gpp(NH)p in the preincubation reduced the Carbo-Am-induced receptor loss to 51% with no change in the K_D value for [^{125}I]CYP (10.8 pM). In one other series of experiments, the ability of various doses of the two derivatives to induce a receptor loss was determined. Incubation of reticulocyte membranes at 32°C for 30 min with 10, 1, 0.1 and 0.01 μM of Carbo-Br followed by four membrane wash cycles reduced specific [^{125}I]CYP binding by 97, 93, 86 and 80%, respectively. In contrast, using the same concentrations of Carbo-Am, the receptor content was reduced by 73, 73, 72 and 62%, respectively.

Table 2-1 showed the effects of several treatments on the ability of the carbostyril derivatives to bind to the beta-adrenoreceptor in reticulocyte membranes. Preincubation of membranes with nadolol (10 μM) followed by washing did not reduce specific [^{125}I]CYP binding indicating that this beta-antagonist is easily washed off the receptor and out of the membranes (unlike propranolol). Furthermore, membrane heating at 45°C for 30 min had no significant effect on the receptor. Preincubation with Carbo-Am (1 μM) followed by membrane washing, reduced the binding by 73%, which could be

Table 2-1. Effects of nadolol and heat treatment on the Carbo-Am and Carbo-Br-induced receptor loss in reticulocyte membranes.

Pretreatment A	Pretreatment B	[^{125}I]CYP Bound (%Control)
-------	-------	100
Nadolol (10 μM)	-------	110 ± 6
-------	45°C	97 ± 4
Carbo-Am (1 μM)	-------	27 ± 3
Carbo-Am (1 μM) plus Nadolol (10 μM)	-------	87 ± 5
Carbo-Am (1 μM)	Nadolol (10 μM)	29 ± 6
Carbo-Am (1 μM)	45°C plus Nadolol (10 μM)	82 ± 4
Carbo-Br (1 μM)	-------	12 ± 4
Carbo-Br (1 μM) plus Nadolol (10 μM)	-------	96 ± 2
Carbo-Br (1 μM)	45°C plus Nadolol (10 μM)	16 ± 2
Carbo-cysteine (1 μM)	--------	25 ± 4
Carbo-cysteine (1 μM)	45°C plus Nadolol (10 μM)	86 ± 6

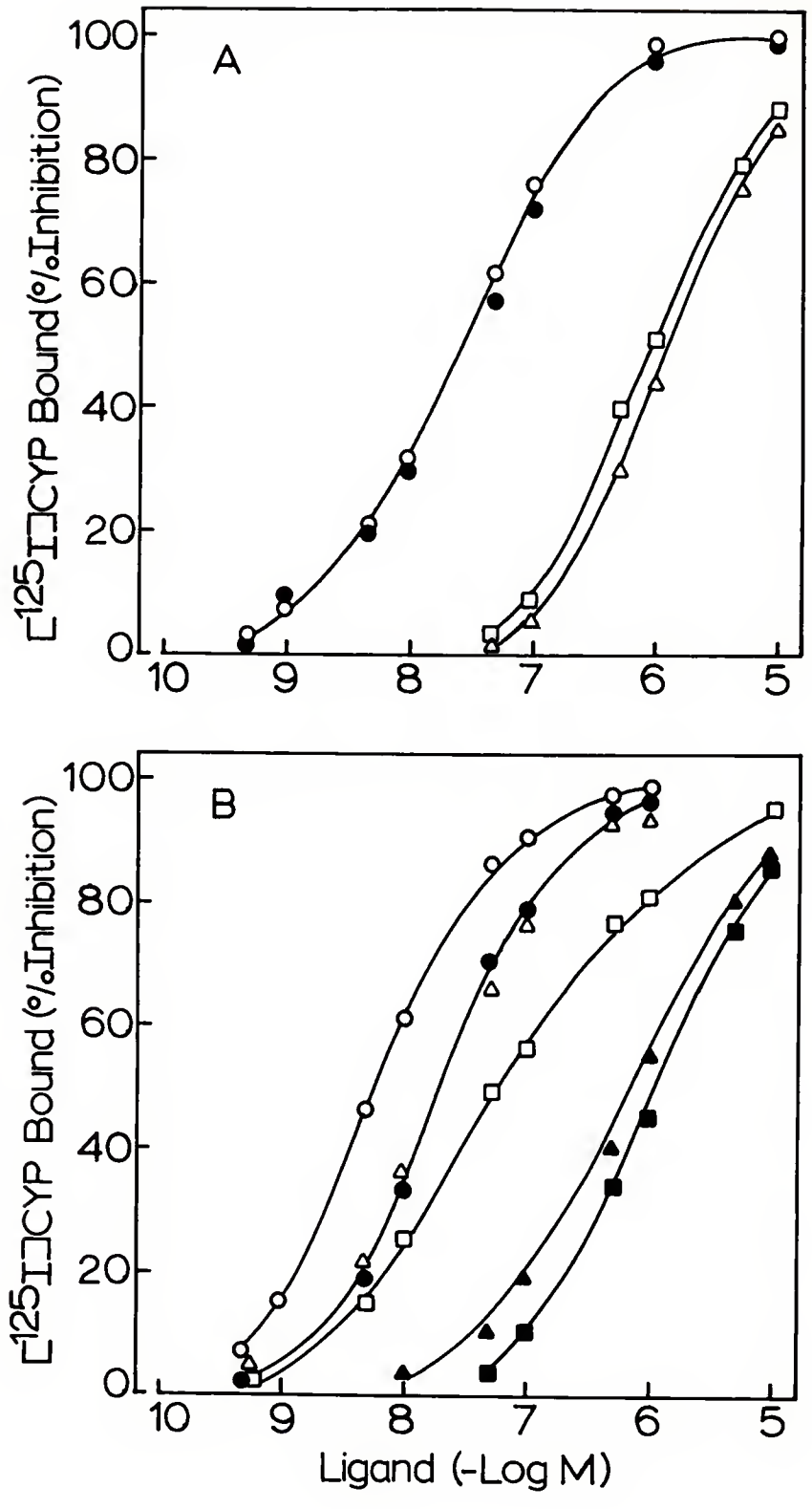
In pretreatment A, membrane protein was incubated with buffer and the additions indicated for 30 min at 32°C. At the end of the incubation, the membranes were washed four times and assayed with [^{125}I]CYP (100 pM). Alternatively, at the end of pretreatment A, the membranes were washed once, resuspended in 50 mM sodium phosphate buffer plus the additions under pretreatment B and incubated for 30 min at 45°C or at 32°C for 20 min in the presence of 10 μM nadolol. At the end of the incubation, the membranes were washed four times with ice-cold buffer and assayed with [^{125}I]CYP at 100 pM. Carbo-cysteine is Carbo-Br (0.5 mM) reacted for 18 hr at 25°C with cysteine (2 mM) and diluted to the final concentration indicated. Values are the mean of triplicate determinations ± S.D, n=3-4.

largely prevented (87% of control) by concurrent incubation with 10 μM nadolol. However, no receptor recovery occurred if the membranes were incubated with 10 μM nadolol after the Carbo-Am pretreatment. The initial Carbo-Am-induced loss of receptors could be substantially recovered (82% of control) by subsequent membrane heating at 45°C in the presence of nadolol. Table 2-1 also showed that membranes pretreated with Carbo-Br (1 μM) followed by washing had an 88% decrease in specific [^{125}I]CYP binding which was largely attenuated by concurrent pretreatment with 10 μM nadolol. When membranes were pretreated with Carbo-Br, washed and then incubated with nadolol at 45°C for 30 min followed by washing, there was little reversal (<10%) of the lost specific binding sites. In contrast, when a solution of Carbo-Br was incubated with cysteine (25°C, 18 hr), diluted to 1 μM and incubated with membranes followed by washing, there was a 75% loss of binding sites. These lost sites were largely recovered (86% of control) if the pretreated membranes were further incubated with nadolol at 45°C for 30 min followed by washing. A diluted sample of cysteine alone had no effect on [^{125}I]CYP binding.

Current evidence indicated that in the absence of a guanine nucleotide, beta-agonists promoted the formation of a ternary complex composed of the agonist, the beta-adreno-receptor and N_S. Agonist binding in the complex was of high affinity. In the presence of a guanine nucleotide, the complex destablized and agonist affinity decreased (De Lean

et al., 1980; Kent *et al.*, 1980). To explore if the small Gpp(NH)p-induced potency shift for the Carbo-Am was due to a tightly bound receptor-N_S complex resistant to guanine nucleotide destabilization, competition experiments were performed under conditions where guanine nucleotide modulation of receptor affinity (receptor-N_S interactions) was greatly reduced. Two preparations were used: 1) rat erythrocyte membranes which have a greatly reduced content of N_S (Limbird *et al.*, 1980a), and 2) reticulo-cyte membranes heated at 50°C to destroy the ability of guanine nucleotides to modulate receptor affinity (Baker *et al.*, 1985). Figure 2-3A showed the ability of Iso and Carbo-Am to inhibit specific [^{125}I]CYP binding in rat erythrocyte membranes. In the absence of Gpp(NH)p, the IC_{50} value for Iso was 941 ± 67 nM. In the presence of 100 µM Gpp(NH)p, there was only a slight increase in the IC_{50} value to 1263 ± 60 nM. The IC_{50} values for the Carbo-Am were not different when the assays were performed in the presence or absence of Gpp(NH)p (IC_{50} values: minus Gpp(NH)p, 27 ± 1.2 nM; plus Gpp(NH)p, 29 ± 2.5 nM). Figure 2-3B showed the competition curves in reticulo-cyte membranes. In control membranes, the IC_{50} value for Iso was shifted 20.4-fold from 58 ± 7.5 nM in the absence of Gpp(NH)p to 1182 ± 20 nM in the presence of 100 µM Gpp(NH)p. After treatment of membranes for 45 min at 50°C, the IC_{50} value for Iso in the absence of Gpp(NH)p was 750 ± 57 nM. The IC_{50} value for Carbo-Am in control membranes and in the absence of Gpp(NH)p was 7.3 ± 0.3 nM which was increased

Figure 2-3. Inhibition of specific [^{125}I]CYP binding in rat erythrocyte (A) and reticulocyte (B) membranes by Iso and Carbo-Am. In A, erythrocyte membranes were incubated with buffer, 30 pM [^{125}I]CYP and the indicated concentrations of Iso (squares), Iso plus 100 µM Gpp(NH)p (triangles), Carbo-Am (open circles) or Carbo-Am plus 100 µM Gpp(NH)p (closed circles) for 45 min at 36°C. In B, control reticulocyte membranes were incubated with buffer, 30 pM [^{125}I]CYP and the indicated concentration of Iso (open squares) and Iso plus 100 µM Gpp(NH)p (closed squares), Carbo-Am (open circles) or Carbo-Am plus 100 µM Gpp(NH)p (closed circles), for 45 min at 36°C. In addition, reticulocyte membranes in 50 mM sodium phosphate buffer at pH 7.4 were incubated at 50°C for 45 min, washed 1 time in buffer and the competition assay carried out with the indicated concentration of Iso (closed triangles) and Carbo-Am (open triangles). In all assays utilizing Iso, 0.1% ascorbate was also present. At the end of the incubations, the specific binding was determined as described in the "Methods" section. Each point on the graph was the mean of three determinations assayed in triplicate. The control [^{125}I]CYP binding values were 39 ± 7, 622 ± 13 and 644 ± 12 fmol/mg protein for the erythrocyte, control reticulocyte and heat-treated reticulocyte membranes, respectively.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to 26 ± 1.1 nM in the presence of Gpp(NH)p. After heat treatment, the IC_{50} value in the absence of Gpp(NH)p was 21 ± 1.5 nM. The IC_{50} values of the Carbo-Am in the presence of Gpp(NH)p in control reticulocytes (26 nM) was virtually identical to the heat-treated membranes (21 nM) in the absence of Gpp(NH)p and erythrocyte membranes both in the presence or absence of Gpp(NH)p (27-29 nM).

Effects of the carbostyril derivatives on reticulocyte adenylate cyclase activity. Figure 2-4 showed the ability of Iso and the two carbostyril derivatives to stimulate cAMP formation. The concentration that produced half-maximal formation was 8.2 ± 2.1, 17.8 ± 3.1 and 241 ± 17 nM for Carbo-Br, Carbo-Am and Iso, respectively. In addition, the maximal formation of cAMP was the same for all 3 compounds. Unlike Iso, both of the carbostyril derivatives appear to be quite stable agonists: even after several weeks in solution they retained full potency and efficacy to stimulate adenylate cyclase. Table 2-2 showed the partial specificity of Carbo-Br in the adenylate cyclase system. In reticulocyte membranes, Forskolin (1 μM) stimulated cAMP production by 17.9-fold. When Iso (10 μM) or Carbo-Br (10 μM) was added in addition to Forskolin, the fold stimulation for both was 23. Using GH_3 cell membranes, forskolin (1 μM) stimulated cAMP formation 15.9-fold. However, Carbo-Br (10 μM) did not stimulate cAMP formation above the basal level. Membranes from GH_3 cells did not appear to contain beta-adrenoreceptors

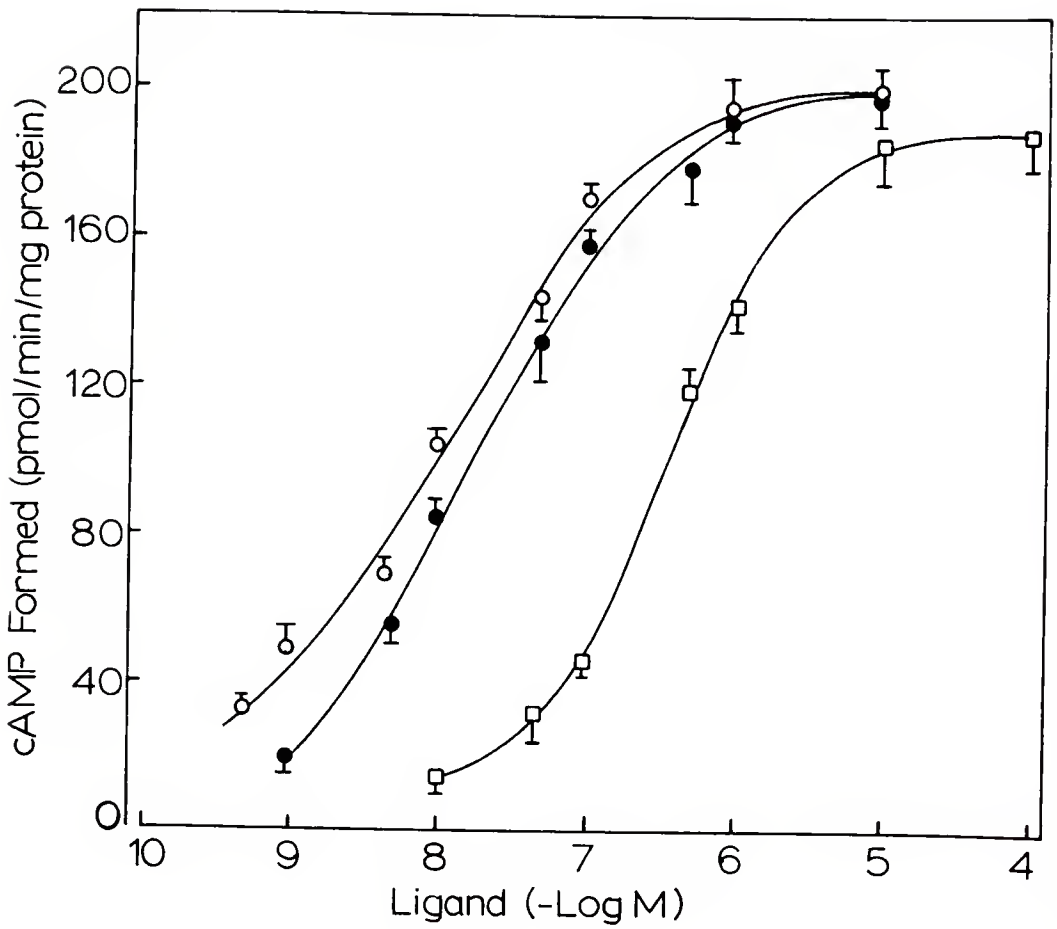


Figure 2-4. Stimulation of reticulocyte adenylate cyclase activity by Iso, Carbo-Am and Carbo-Br. Cyclase activity was determined by incubating membrane protein (35 µg) in buffer pH 7.4 containing 500 µM GTP, the indicated concentration of Iso (squares), Carbo-Am (closed circles) and Carbo-Br (open circles) for 10 min at 32°C. Other standard cyclase assay components and the determination of the cAMP content were performed as described in the "Methods" section. Each data point is the mean ± S.D., n = 3. Basal activity in the presence of GTP was 17 ± 4 pmol/min/mg protein and was subtracted from the stimulated values.

Table 2-2. Effect of Carbo-Br on adenylate cyclase activity in rat reticulocyte and GH_3 cell membranes.

Additions	cAMP Formed (pmol/min/mg protein)
A Reticulocytes	
Basal	14.7 ± 1.4
Forskolin (1 μM)	264 ± 8.0
Isoproterenol (10 μM) plus Forskolin (1 μM)	347 ± 11.0[a]
Carbo-Br (10 μM) plus Forskolin (1 μM)	339 ± 9.0[a]
B. GH_3 membranes	
Basal plus GTP	10.0 ± 3.3
Forskolin (1 μM)	159.0 ± 11.0
Carbo-Br (10 μM) plus GTP (500 μM)	9.6 ± 1.4

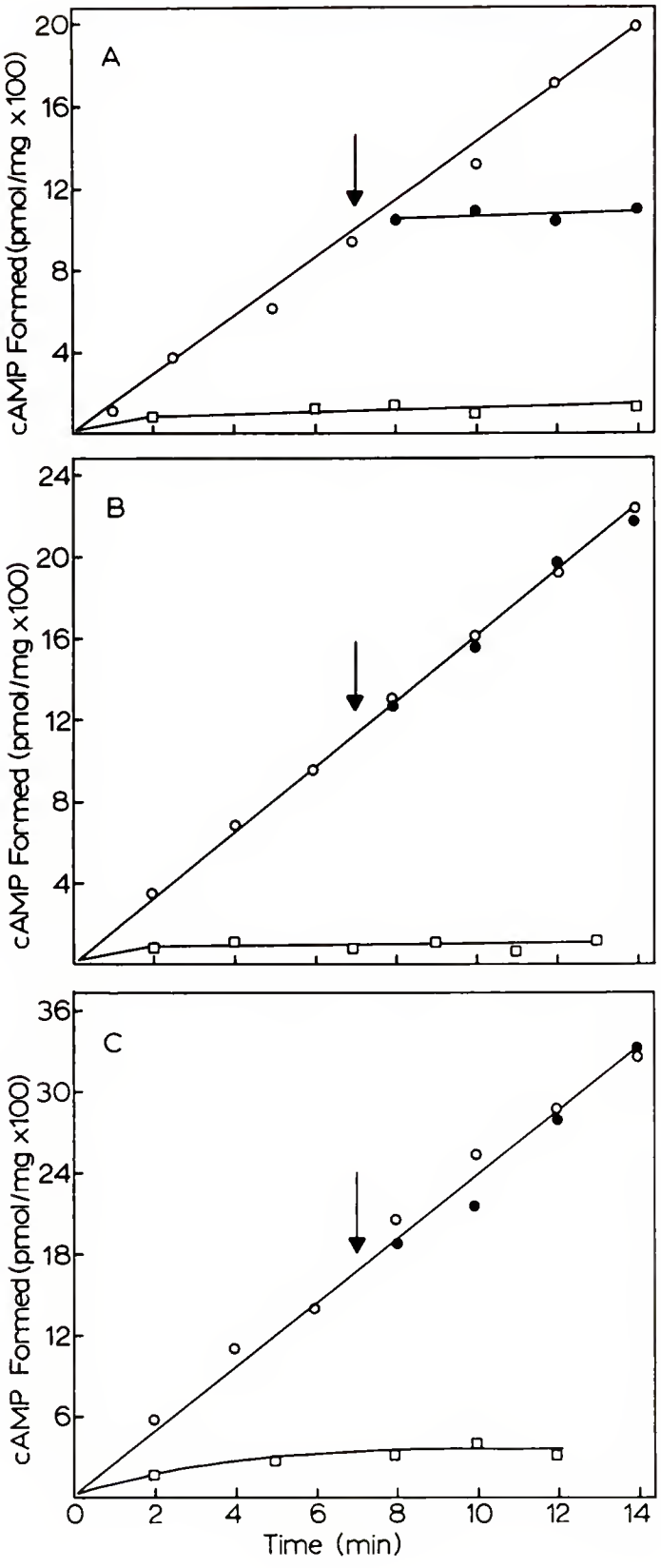
Membrane protein (reticulocytes, 35 μg; GH_3, 50 μg) was incubated with the standard cyclase assay components and the additions indicated for 10 min at 32°C. At the end of the incubation, the cAMP formed was determined as described under "Experimental Procedures". Values are the means of triplicate determinations ± S.E., n=3-4.

[a]Significantly different from the Forskolin group ($p < 0.01$).

as no specific [^{125}I]CYP (3-100 pM) binding was detectable (data not shown).

The time course of cAMP formation in reticulocyte membranes induced by Iso (10 μM), Carbo-Br (1 μM) and Carbo-Am (1 μM) was shown in Figures 2-5A, B and C, respectively. Cyclic AMP formation in the presence of all three compounds was linear for at least 14 min of incubation. When propranolol (20 μM) was added after 7 min of incubation, the Iso-stimulated formation of cAMP was blocked (Figure 2-5A). However, when propranolol (20 μM) was added after 7 min of incubation with Carbo-Br (Figure 2-5B) or Carbo-Am (Figure 2-5C), no effect on the rate of cAMP formation was observed. In contrast, if propranolol was added at time zero with the three compounds, then cAMP formation by all three was largely blocked. Figure 2-6 showed the time course of Carbo-Am and Carbo-Br-induced receptor loss in reticulocyte membranes. Incubations were carried out at 32°C with 1 μM of both compounds plus all of the additions used in the assay of adenylate cyclase activity. At various times during the incubation, membrane samples were washed four times and assayed for beta-adrenoreceptors. After 2 min of incubation, specific [^{125}I]CYP binding was decreased by 51 and 61% in membranes incubated with Carbo-Am and Carbo-Br, respectively. This loss continued slowly until, by the end of 12 min, specific binding sites had decreased 61% in the Carbo-Am treated membranes and 75% in those treated with

Figure 2-5. Time course of reticulocyte adenylate cyclase activation by Iso (A), Carbo-Am (B) and Carbo-Br (C). Cyclase activity was determined by incubating membrane protein (35 µg) in buffer containing 500 µM GTP, 10 µM Iso (A), 1 µM Carbo-Br (B) or 1 µM Carbo-Am (C) at 32°C. Activity was determined with the agonists alone (open circles), agonists plus 20 µM propranolol added at time 0 (squares) or after 7 min of incubation with the agonists alone, propranolol (20 µM) was added (closed circles). The arrow indicated the time when propranolol was added. Other standard cyclase components and the determination of the cAMP content were as described in the "Methods" section. Each data point was the mean of triplicate determinations and was representative of four experiments. Basal cAMP formation in the presence of GTP alone was subtracted from the stimulated values.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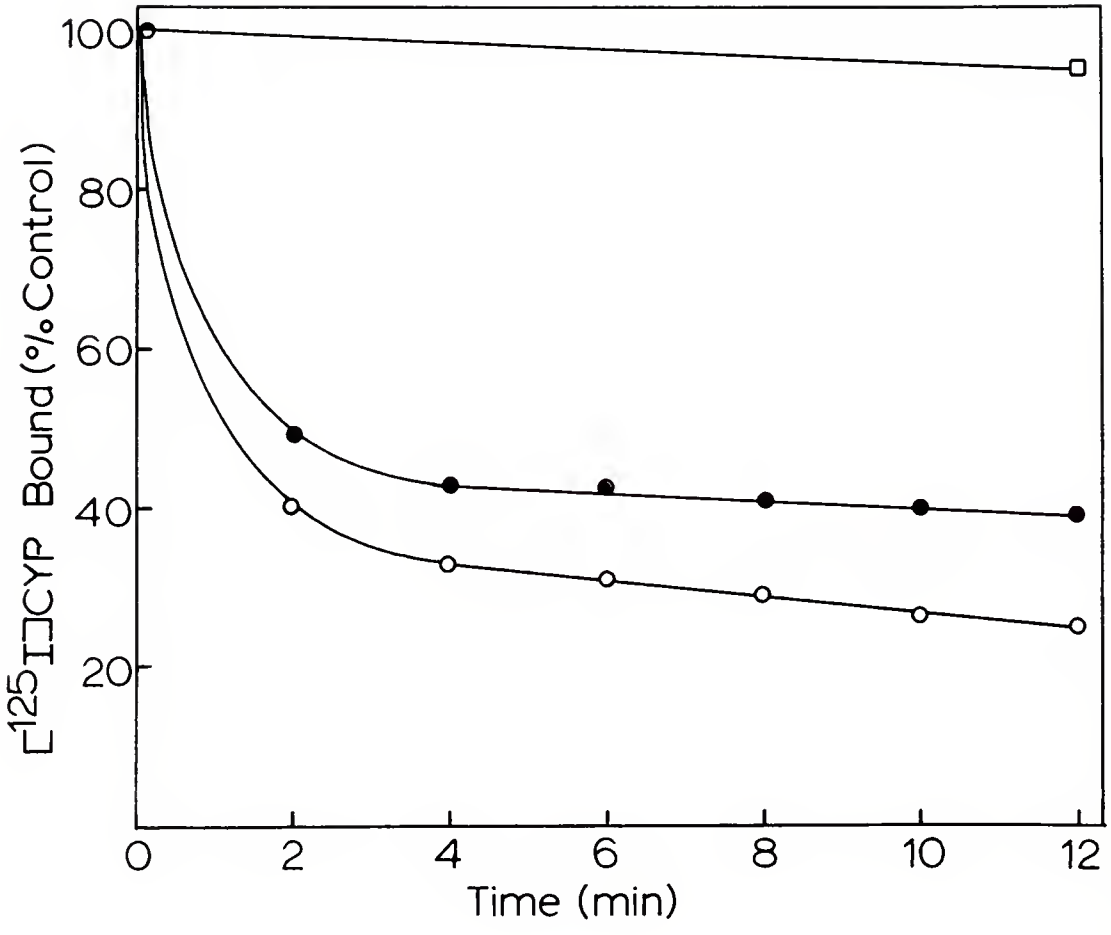

Figure 2-6. Time course of specific [^{125}I]CYP binding loss by Carbo-Am and Carbo-Br in reticulocyte membranes. Membrane protein (0.26 mg/ml) was incubated in buffer containing 1.6 mM ATP, 500 µM GTP, 1.0 mM EGTA, 10 mM theophylline, 0.1% BSA, creatine phosphokinase (67 units/ml), 2.5 mM phosphocreatine and without (squares) and with 1 µM Carbo-Am (closed circles) or 1 µM Carbo-Br (open circles) at 32°C. At the times indicated, samples were removed, the membranes washed four times with ice-cold buffer and assayed with 100 pM [^{125}I]CYP as described in the "Methods" section. Data points are the mean of three determinations. The control [^{125}I]CYP binding values were 566 ± 17 fmol/mg of protein in the Carbo-Am experiments and 575 ± 25 fmol/mg protein in the Carbo-Br experiments.

Carbo-Br. Less than 5% of the binding sites were lost over the 12 min incubation period in control membranes.

Discussion

The data from this study showed that both of the carbostyril derivatives were highly potent beta-adrenergic agonists. The Carbo-Am and Carbo-Br compounds were 14- and 29-fold, respectively, more potent than Iso in stimulating adenylate cyclase activity. That enzyme activation by the carbostyril derivatives was mediated through the beta-adrenoreceptor rather than a nonspecific effect was indicated by two lines of evidence. First, concurrent addition of propranolol, a beta-antagonist, blocked the enzyme activation by both compounds. Second, the enzyme in GH_3 cell membranes was vigorously stimulated by forskolin but not by Carbo-Br. These membranes did not contain any detectable beta-adrenoreceptors (Henneberry *et al.*, 1986). The observation that both of the carbostyril congeners produced the same maximal stimulation of adenylate cyclase activity as Iso indicated that both compounds were full beta-agonists.

Current evidence has suggested that a beta-agonist, the beta-adrenoreceptor and N_S interact to form a ternary complex (Citri and Schramm, 1980; De Lean *et al.*, 1980; Kent *et al.*, 1980; Limbird *et al.*, 1980b). When a guanine nucleotide has bound to N_S, it destabilized the ternary complex, causing N_S to dissociate from the receptor and reduced the receptor

affinity for the agonist which can then dissociated from the receptor. Thus, in the absence of a guanine nucleotide, a substantial fraction of the beta-adrenoreceptors showed high affinity for the receptor (ternary complex formation) whereas in the presence of a guanine nucleotide all of the receptors showed an agonist low affinity binding state (Lefkowitz *et al.*, 1976; Maguire *et al.*, 1976; De Lean *et al.*, 1980). The initial ternary complex formation appeared to be a necessary prerequisite for a beta-agonist to stimulate adenylate cyclase activity (De Lean *et al.*, 1980; Kent *et al.*, 1980). From competition studies in the absence of guanine nucleotide, the Carbo-Am and Carbo-Br compounds were 8- and 14-fold more potent than Iso. In the presence of Gpp(NH)p, however, the Carbo-Am and Carbo-Br were 39- and 106-fold (respectively) more potent than Iso. This substantial increase in the difference in potency between the carbostyril derivatives and Iso in the presence of guanine nucleotides was apparently due to the large Gpp(NH)p-induced decrease in the affinity of Iso for the receptor (17-fold) whereas Gpp(NH)p reduced the potency of the carbostyril compounds only slightly (2- to 3.5-fold).

It has been reported that there is a direct relationship between the ability of a beta-agonist to stimulate maximally adenylate cyclase activity (intrinsic activity) and the Gpp(NH)p-induced shift in agonist affinity (Lefkowitz *et al.*, 1976; Kent *et al.*, 1980). Full agonists have large affinity shifts whereas partial agonists have small affinity shifts.

Since both of the carbostyril congeners act as full agonists with relatively small Gpp(NH)p-induced potency shifts, they would appear to be exceptions to the above relationship. However, there were several possible reasons for the small Gpp(NH)p-induced shift in potency for these compounds. These agonists could promote an extremely tight receptor-N_s complex such that even in the presence of a guanine nucleotide little complex destabilization (and hence a reduction in agonist potency) is observed. If this is correct, then the loss of N_s should reduce the potency beyond that observed in the presence of Gpp(NH)p. However, this possibility was unlikely since the IC_{50} values for Carbo-Am were very similar using control reticulocyte membranes in the presence of Gpp(NH)p, using reticulocyte membranes treated at 50°C (to reduce functional N_s) [Baker *et al.*, 1985], and using erythrocyte membranes (which lack N_s) [Limbird *et al.*, 1980a]. An alternative explanation for the small Gpp(NH)p-induced shift could be an extremely tight (quasi-irreversible) or irreversible (covalent) binding of the carbostyril derivatives to the receptor. Previous studies have shown that the major effect of guanine nucleotides is to markedly increase the dissociation rate of the agonist from the receptor (Williams & Lefkowitz, 1977; Heidenreich *et al.*, 1980). Thus an extremely tight binding or covalent attachment of the agonist to the receptor might greatly reduce or prevent the Gpp(NH)p-induced agonist dissociation resulting in a small potency

shift. As discussed below, this was likely for the carbostyril derivatives.

Several lines of evidence suggested that the carbostyril derivatives bound in an extremely tight and/or irreversible manner to the beta-adrenoreceptor. Scatchard analysis of [^{125}I]CYP binding after incubation with Carbo-Br or Carbo-Am and membrane washing indicated a quasi-irreversible interaction as the receptor capacity decreased, whereas the K_D for [^{125}I]CYP binding to the remaining receptors did not change. In keeping with the small Gpp(NH)p-induced shifts to lower potency for both compounds, the inclusion of Gpp(NH)p during the membrane preincubation reduced the receptor loss to a relatively small degree. Furthermore, the receptor loss induced by the carbostyril derivatives was largely attenuated by concurrent incubation with nadolol. These results suggested that the initial interaction of both carbostyril derivatives with the receptor was competitive followed by a longer term quasi-irreversible binding. The Carbo-Am compound contained no highly reactive moiety, suggesting that its tight binding to the receptor was noncovalent in nature. This was supported by the finding that a substantial reversal of Carbo-Am binding occurred (>50%) by incubation of membranes at 45°C in the presence of nadolol. This effect may have been due to a high temperature-induced dissociation of the ligand from an intact receptor structure or a dissociation induced by a reversible denaturation of the receptor at 45°C. A quasi-irreversible binding of two

beta-adrenoreceptor antagonists has been previously reported (Terasaki *et al.*, 1979; Lucas *et al.*, 1979). In contrast to the Carbo-Am, the Carbo-Br compound contained a reactive bromoacetyl moiety. Thus its binding to the receptor may have involved a loss of the bromo group resulting in a reactive electrophilic ligand that could undergo an irreversible alkylation of a nucleophile in the receptor. This was supported by the observation that only a small reversal of Carbo-Br binding (<10%) occurred by membrane heating at 45°C. Furthermore, after reacting Carbo-Br with cysteine to inactivate the bromoacetyl moiety, a relatively large reversal (58%) of its (carbo-cysteine) binding to the receptor was found after membrane heating at 45°C. Although these data were consistent with a covalent attachment of Carbo-Br to the receptor, another alternative was that Carbo-Br bound reversibly with even higher affinity than Carbo-Am such that little dissociation occurred, even at 45°C. Definitive proof of a covalent interaction would require more experiments, perhaps using a radiolabelled compound in conjunction with purified receptors as previously described for an alkylating antagonist (Dickinson *et al.*, 1985). Interestingly, the maximal receptor loss induced by a wide range of Carbo-Am concentrations was about 75%, suggesting that a small fraction of receptors was resistant to the tight binding of this compound. In contrast, the maximal receptor loss induced by Carbo-Br approached 100%.

The data from the time course of reticulocyte adenylate cyclase activation suggested that the two carbostyril compounds acted as quasi-irreversible agonists. This was indicated by the observation that addition of a 20-fold excess of propranolol after seven min of incubation with the two derivatives alone did not affect the rate of cAMP accumulation. During the first seven min of incubation alone with Carbo-Am or Carbo-Br, there was a 59 and 71% reduction in specific [^{125}I]CYP binding sites, respectively. In contrast, when propranolol was added after seven min of incubation with Iso alone, there was a complete blockade of further cAMP production, consistent with Iso being a fully reversible agonist. In addition, when propranolol was added concurrently with both of the carbostyril compounds and Iso, stimulation of the enzyme was virtually prevented. Thus the cyclase activation data in conjunction with the previously discussed binding data indicated that the initial interaction of the carbostyril derivatives with the beta-adrenoreceptor was competitive followed by a longer term quasi-irreversible interaction. The activation of receptors by agonists has been explained by two basic theories. The occupation theory involved receptor activation as long as the receptor was occupied whereas the rate theory predicted that activation was proportional to the rate of combination between the receptor and agonist (see Bowman & Rand, 1980; Yamamura *et al.*, 1985 for reviews). The data showing an apparent irreversible activation of adenylate cyclase by the carbostyril

congeners supported the occupancy theory for the beta-adrenergic system.

In comparison to the apparent irreversible agonist effects of the carbostyril derivatives, we recently reported that bromoacetylaminomenthylnorepinephrine (BAAN) could, under defined conditions, bind to the beta-adrenoreceptor in an irreversible manner (Baker *et al.*, 1985). Although BAAN initially stimulated adenylate cyclase activity, after irreversible binding it acted as an antagonist. The reasons for the lack of an irreversible agonist effect by BAAN whereas the carbostyril derivatives produced an apparent irreversible agonist action were not obvious. These differences might be related to the structures of the compounds whereby different ligand-induced conformational changes in the receptor were produced. Alternatively, the differences might be related to the observation that BAAN was a weak partial agonist whereas the carbostyril derivatives were potent full agonists. Further studies would be necessary to delineate the apparent irreversible effects of these compounds. Although, to our knowledge, this was the first report describing an apparent irreversible agonist for the beta-adrenergic system, ligands showing irreversible or sustained agonist effects have been reported for the insulin (Brandenburg *et al.*, 1980), adrenocorticotropin (Ramachandran *et al.*, 1981), adenosine (Lohse *et al.*, 1986), and opiate (Schoenecker *et al.*, 1987) receptors.

In summary, the experimental results indicated that the two carbostyril derivatives were stable, potent full beta-adrenergic agonists that produced sustained activation effects *in vitro*. The tight binding of the Carbo-Am to the beta-adrenoreceptor appeared to be noncovalent whereas the quasi-irreversible binding of the Carbo-Br might have involved receptor alkylation.

CHAPTER 3
INTACT CELL STUDIES

Introduction

All of the experiments described thus far were performed on rat reticulocyte membranes. This preparation offered a simplified, cell-free system within which various components could be easily manipulated. Some interesting results pertaining to the interactions of the carbostyril congeners with beta$_2$-adrenoreceptors were obtained through examination of this simplified system. Nonetheless, beta-adrenoreceptor agonists and antagonists have been shown to interact *in vivo* with living cells, not membrane fragments (Abramson & Molinoff, 1984). Beta-adrenoreceptors on intact cells possessed properties not found in simple membrane preparations. One example of this was the agonist-induced process of desensitization. The desensitization sequence began with a rapid uncoupling of cell surface beta-adrenoreceptors from stimulation of adenylate cyclase (Su *et al.*, 1980; Harden *et al.*, 1979). This was followed by an apparent internalization or sequestration of the beta-adrenoreceptor from the cell surface (Toews *et al.*, 1984; Toews & Perkins, 1984; Harden *et al.*, 1980), and eventual down-regulation (loss of binding function) of total receptor number (Su *et*

al., 1980). The ability of internalized receptors to recycle back to the cell surface depended on the length of exposure to the agonist. In most cases (i.e., acute agonist pretreatment), when the agonist was removed, receptors would reappear on the cell surface without further protein synthesis (Hertel & Staehelin, 1983; Stadel *et al.*, 1983; Doss *et al.*, 1981). In contrast, after chronic agonist treatment, receptors were internalized and degraded. Therefore, because of the differences between an intact cell system and isolated membranes, we have characterized the agonist-induced changes of Carbo-Br in the DDT_1-MF-2 smooth muscle cell line. The ability of Carbo-Br to bind to $alpha_1$-adrenoreceptors present in DDT membranes was examined also. Evidence was provided to indicate that while Carbo-Br bound either extremely tightly or irreversibly, it still caused a desensitization effect in the same manner as Iso.

Experimental Procedures

Source of Materials

The radioligands (-)-[^{3}H]CGP-12177 ([^{3}H]CGP; 38.8-53.1 Ci/mmol) and [^{3}H]Prazosin (82 Ci/mmol) were obtained from New England Nuclear, Boston, MA, USA. The antagonist CGP-20712A was a generous gift from Dr. L. Maitre at Ciba-Geigy, Ltd., Basle, Switzerland. Three-isobutyl-one-methylxanthine (IBMX), and snake venom (*crotalus atrox*, western diamondback rattlesnake) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Dowex 1-X8 anion exchange resin (200-400 mesh, chloride form) was purchased from Bio-Rad Laboratories,

Richmond, VA, USA and 2-mercaptoethanol was a gift from Dr. S.R. Childers, University of Florida. Cultured cell lines DDT_1-MF-2 and C6 were obtained from American Type Culture Collection, Rockville, MD, USA. The C62B cell line was a gift from Dr. Mark Rasenick, University of Illinois College of Medicine.

Methods

Buffer. Studies with intact cells were carried out using Hank's Balanced Salt Solution (HBSS) unless otherwise noted. The solution contains 5 mM KCl, 0.4 mM KH_2PO_4, 137 mM NaCl, 4 mM $NaHCO_3$, 0.6 mM Na_2HPO_4, 6 mM D-glucose, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, and 1 mM $CaCl_2$, pH 7.4.

Cell culture. The DDT_1-MF-2 cell line (a smooth muscle cell line derived from a leiomyosarcoma of the ductus deferens of Syrian hamster) and both lines of C6 cells (from rat glial tumors) were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2.75 µg/ml amphotericin B, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin sulfate in a humidified atmosphere of 95% air/5% CO_2. Cells were routinely subcultured every seven days with trypsin (0.01%) from an initial inoculum of 4-6 x 10^5 c/ml.

Cell harvesting and treatment. Cells were harvested at approximately 50% confluence. Cell monolayers were washed twice with PBS to remove culture medium. Cells used in intact cell studies were incubated for 5 min at 36°C in 3-10 ml of PBS containing 1 mM EGTA. The cells were lifted with repeated pipetting, centrifuged at 1000xg for 5 min, and

resuspended in HBSS. In pretreatment studies, cells were incubated for various times in the presence or absence of 10 μM Iso or 1 μM Carbo-Br (final concentrations) at 36°C. Cells were then washed five times with cold HBSS by centrifugation at 1000xg followed by resuspension. Whole cell homogenates were obtained by resuspending the final pellet in 50 mM Tris-HCl pH 7.4 containing 5 mM $MgCl_2$ and homogenizing for 15 sec at setting 2.5. Binding assays were performed as described above with [^{125}I]CYP using 0.5 mg/ml protein.

Antagonist Binding Assays. Beta-adrenoreceptor content of DDT and C6 membranes was determined by incubating membrane protein (25 μg) in a total volume of 0.25 ml with 50 mM Tris-HCl at pH 7.4, 5 mM $MgCl_2$, 3-100 pM [^{125}I]CYP and with and without 1 μM (±)alprenolol for 60 min at 36°C. At the end of the incubation, each suspension was diluted with 3 ml of 50 mM Tris-HCl pH 7.4 (36°C) and poured onto a Whatman GF/B glass fiber filter under reduced pressure. The filter was washed with a further 6 ml of buffer, placed in a vial and the radioactivity determined. Specific [^{125}I]CYP binding to the beta-adrenoreceptor was calculated as the difference between the total binding in the absence of (±)alprenolol and the nonspecific binding determined in the presence of 1 μM (±)alprenolol. Nonspecific binding was the same if (±)alprenolol was replaced with 100 μM Iso. Specific binding was 90-95% of the total bound.

In some experiments, the ability of the carbostyril congeners, Iso, or the beta$_1$-selective antagonist CGP-20712A

to inhibit specific [^{125}I]CYP binding was determined. Assays were the same as above except the [^{125}I]CYP concentration was 30 pM and 0.1% sodium ascorbate was included when Iso was used. The competitive binding assays were also performed in the presence and absence of 100 μM Gpp(NH)p, a non-hydrolyzable analog of GTP. All binding assays were performed in triplicate, the results varying by less than 5%.

The recently introduced beta-antagonist CGP-20712A has been shown to have an extremely high selectivity (about 10,000-fold) for the beta$_1$-adrenoreceptor subtype (Dooley & Bittinger, 1984; Lemoine *et al.*, 1985). In competition assays with a nonselective labelled ligand, low concentrations of CGP-20712A bound to the beta$_1$-subtype. As the concentration of CGP-20712A was increased, a plateau region appeared where no further displacement of labelled ligand occurred until a high enough concentration of CGP-20712A was present to displace binding of the labelled ligand to the beta$_2$-subtype. The ratio of beta$_1$ to beta$_2$ receptors could be estimated from the plateau region of the inhibition curve or calculated using various curve-fitting programs for the analysis of multiple site binding data.

Beta-adrenoreceptor content on the surface of intact DDT cells were measured by incubating cells (0.2 mg/tube) in a total volume of 1.0 ml containing HBSS, 0.2 to 6.3 nM [^{3}H]CGP, and in the presence and absence of 1 μM (±)alprenolol for 2 hr at 4°C. At the end of the incubation, 4 ml of 50 mM Tris-HCl pH 7.4 containing 5 mM $MgCl_2$ at 4°C

was added to each tube, and the suspension was poured onto a GF/C glass fiber filter under reduced pressure. Filters were washed quickly with an additional 8 ml of the same buffer, placed in scintillation vials with 7 ml of Liquiscint and the radioactivity determined. Specific ligand binding was calculated as previously described.

In some experiments, intact cells, in suspension (2 mg/ml) or plated on 143 cm^2 plates, were pretreated for various times with 10 μM Iso or 1 μM Carbo-Br at 36°C. The cells (or plates) were promptly removed to ice, washed five times with HBSS at 4°C, and resuspended in HBSS (cells) or reinoculated in fresh media (plates). The suspended cells were then assayed with a single concentration of [^{3}H]CGP (0.75 nM) as previously described.

Alpha$_1$-adrenoreceptors were measured by incubating membrane protein (0.4 mg/tube) in a total volume of 2.0 ml of 50 mM Tris-HCl pH 7.4, containing 5 mM $MgCl_2$, 0.25 nM [^{3}H]Prazosin, and in the presence or absence of 10 μM phentolamine for 30 min at 30°C. At the end of the incubation, 4 ml of 50 mM Tris-HCl pH 7.4, containing 5 mM $MgCl_2$ at 4°C was added to each tube, and the suspensions poured onto GF/C glass fiber filters under reduced pressure. Filters were washed quickly with an additional 8 ml of the same buffer, placed in scintillation vials with 7 ml Liquiscint and the radioactivity determined. Specific ligand binding was determined as described earlier.

Measurement of cAMP production in intact cells. Intact cell cAMP accumulation was measured by incubating detached cells with various concentrations of Carbo-Br or Iso for 5 min at 36°C in HBSS that contained 0.5 mM of the phosphodiesterase inhibitor IBMX. At the end of the incubation, cells were quickly (20 sec) sedimented by centrifugation at 1000xg to remove the drug as well as any extracellular cAMP. The supernatant was aspirated, the pellet resuspended in 25 mM Tris-HCl buffer (pH 7.0, 4°C), and samples placed in a boiling water bath for 5 min. After boiling, samples were centrifuged at 1000xg and the supernatant saved for cAMP assay as described in Chapter two. For the time course of cAMP accumulation or phosphodiesterase activity assay, cells were incubated with either 1 μM Carbo-Br or 10 μM Iso for various times in the absence of IBMX. The phosphodiesterase inhibitor was added to some cell suspensions after the incubation and before the first spin to prevent further cAMP degradation.

DDT Membrane Adenylate Cyclase Assay. Activity was determined as described earlier in Chapter two "Methods" except that the membrane protein assayed was 65 μg/tube. "Basal" conditions included membrane protein and the regenerating system which included ATP, creatine phosphokinase, phosphocreatine, EGTA, BSA, and theophylline. "Carbo-Br" conditions included basal additions plus GTP and Carbo-Br. "Propranolol" conditions included Carbo-Br additions plus

propranolol. The cAMP content of the supernatant was determined as described in Chapter two, "Methods".

Cyclic AMP Phosphodiesterase Assay. The activity of cAMP phosphodiesterase was examined by utilizing the assay procedure of Thompson & Appleman (1971) with modifications by Meeker & Harden (1982). With all additions made on ice, whole DDT cell homogenates (300 µg/200 µl) were added to tubes containing 20 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 60 µg bovine serum albumin, 0.5-400 µM cAMP, and 100,000 cpm/assay of [^{3}H]cAMP. All dilutions from stock were made with 40 mM Tris-HCl (pH 7.4 at 30°C). The total assay volume was 0.4 ml.

After incubation at 30°C for 20 min, the reaction was terminated by transferring the tubes to a boiling water bath for 2 min. The tubes were then placed in a 4°C bath where 100 µl of snake venom toxin (1 mg/ml) was added. The tubes were incubated for another 10 min at 30°C, and transferred back to the 4°C bath were the incubation was stopped by the addition of 1.0 ml of a 1:2 slurry of Dowex 1-X8. The tubes were mixed by vortexing and the Dowex sedimented by centrifugation for 5 min at 1000xg. An aliquot of 0.5 ml was taken from each tube, added to 9.5 ml Liquiscint and counted for 5 min. Blanks were determined in the presence of boiled protein.

Data analysis. The competition data using CGP-20712A were analyzed both as a one- and two-site model with a "By Hand" curve-fitting program for the analysis of multiple site

radioligand binding data (Richardson & Humrich, 1984). This analysis provided estimates of the competitor's affinity for and the concentration of the two sites. The best fit of the experimental data was obtained as defined by a minimum deviation. The analysis assumed the ligand binding to both sites followed mass-action kinetics. Arguments supporting this assumption have been presented by Minneman *et al.* (1979). Statistical analysis of all other data was performed using the Student's t-test and was presented as mean ± S.E.

Results

Inhibition of specific [^{125}I]CYP binding in DDT membranes by Carbo-Br and Iso. Figure 3-1 showed the ability of Iso and Carbo-Br to inhibit specific binding of [^{125}I]CYP to DDT cell membranes in the presence of 0.1 mM Gpp(NH)p. The IC_{50} values for Carbo-Br and Iso were 9 ± 2 (n=3) and 400 ± 23 nM (n=3), respectively.

Effects of Carbo-Br on DDT cell cAMP production and phosphodiesterase activity. Figure 3-2 showed the ability of Iso and Carbo-Br to stimulate cAMP accumulation in intact DDT cells. Carbo-Br was 9-fold more potent then Iso at stimulating cAMP accumulation with an EC_{50} value of 9 ± 1.6 nM (n=3) compared to 80 ± 12 nM (n=3) for Iso. A time course of cAMP accumulation in DDT cells induced by 1 μM Carbo-Br or 10 μM Iso was shown in Figure 3-3. The time of cAMP accumulation by both agonists was the same over the 60 min time period. After 3 min of incubation there was an

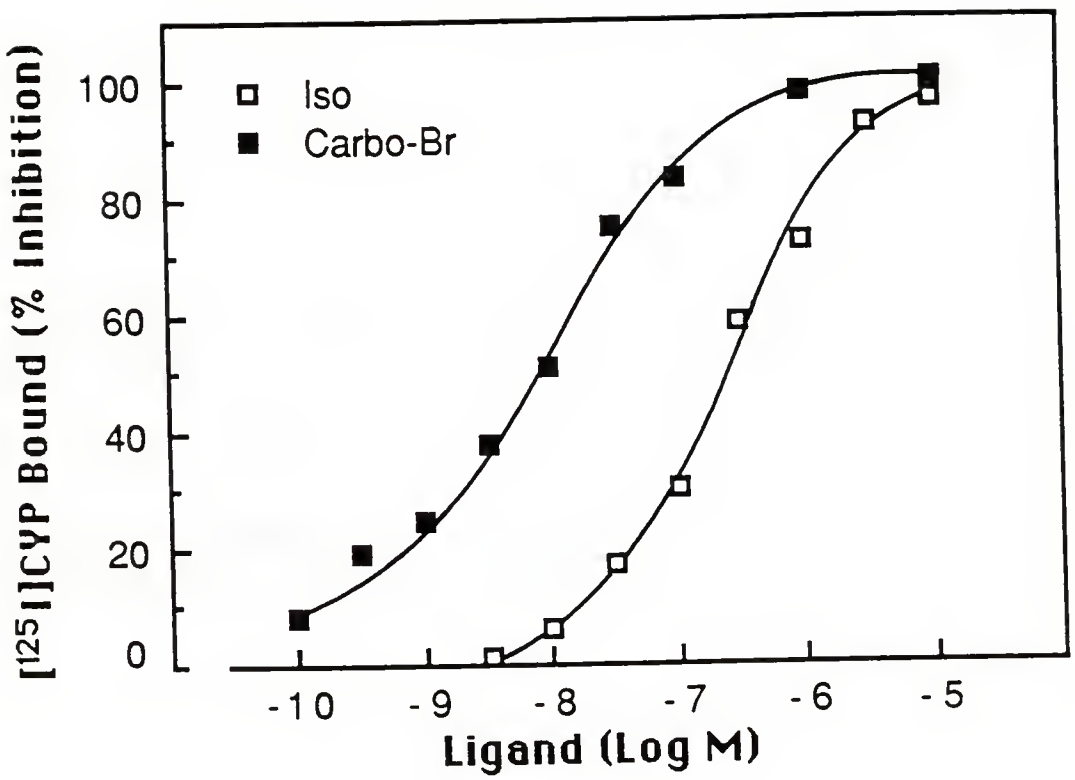

Figure 3-1. Inhibition of specific [^{125}I]CYP binding in DDT cell membranes by Iso and Carbo-Br. Membranes (25 µg/tube) were incubated with buffer at pH 7.4, 30 pM [^{125}I]CYP, 100 µM Gpp(NH)p, and the indicated concentrations of Iso and Carbo-Br for 45 min at 36°C. In the Iso competition assays, 0.1% ascorbate was also present. At the end of the incubation, the specific binding was determined as described under "Methods". Each point on the graph was the mean of 3 determinations assayed in triplicate. Specific [^{125}I]CYP binding ranged from 63 to 69 fmol/mg protein.

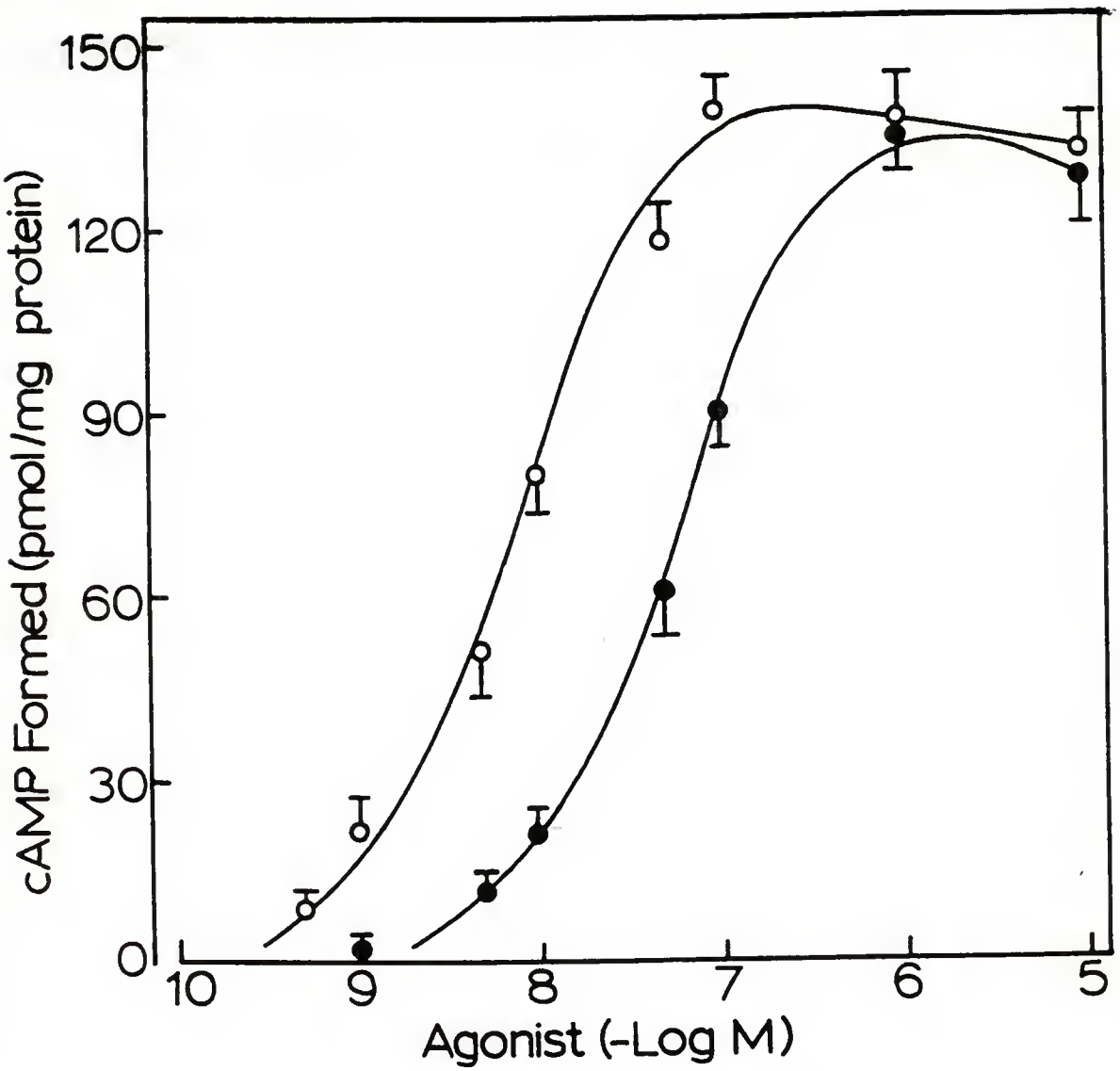

Figure 3-2. Stimulation of cAMP production in intact DDT cells by Iso and Carbo-Br. Cyclic AMP production was determined by incubating intact cells (0.36 mg) in HBSS buffer containing the indicated concentrations of Iso (closed circles) or Carbo-Br (open circles) for 5 min at 36°C. The determination of cAMP content was performed as described under "Methods". Each data point was the mean + S.E., n=3. Basal activity was 10 pmol/min/mg protein and was subtracted from the stimulated values.

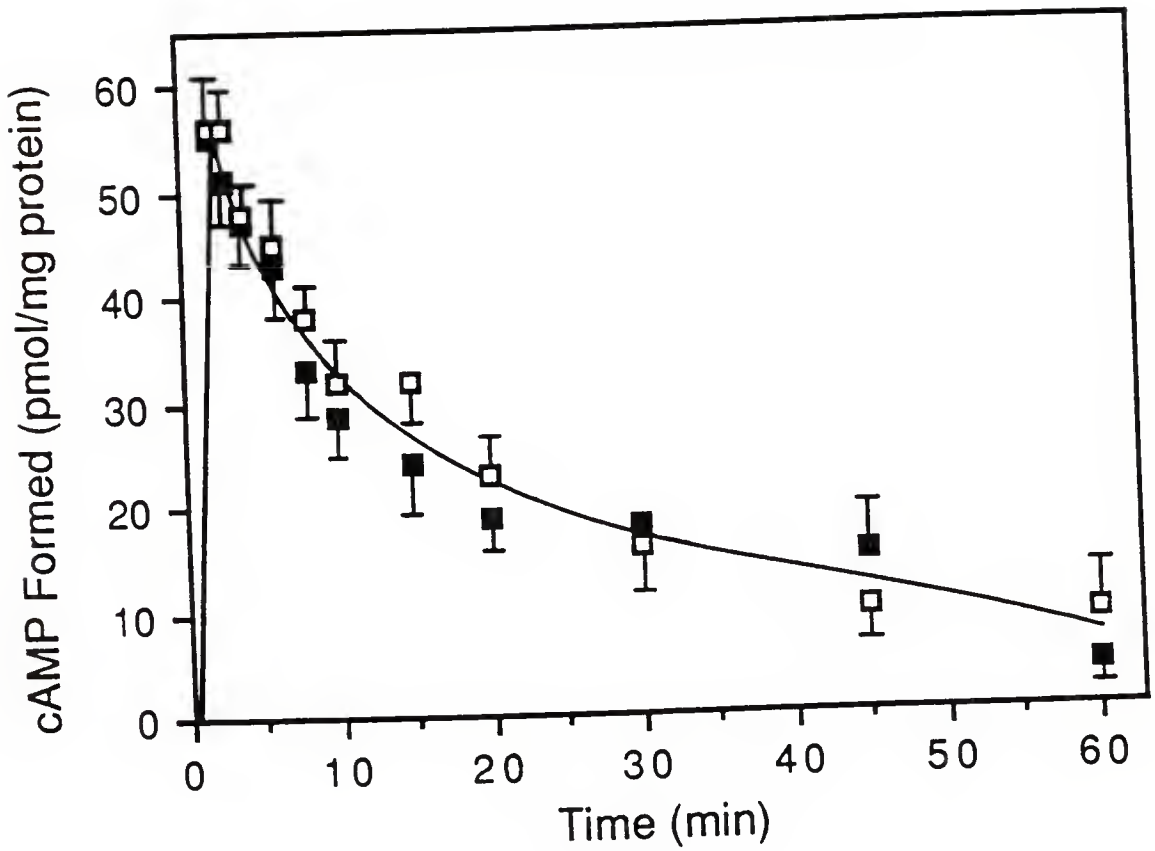

Figure 3-3. Time course of cAMP accumulation in intact DDT cells by Iso and Carbo-Br. Cyclic AMP accumulation was determined by incubating intact cells (0.36 mg) in HBSS buffer containing 10 μM Iso (closed squares) or 1 μM Carbo-Br (open squares) at 36°C for the indicated times. Determination of cAMP was performed as described under "Methods". Each data point was the mean of 3-4 determinations ± S.E. Basal cAMP formation (10 pmol/min/mg protein) has been subtracted from the stimulated values.

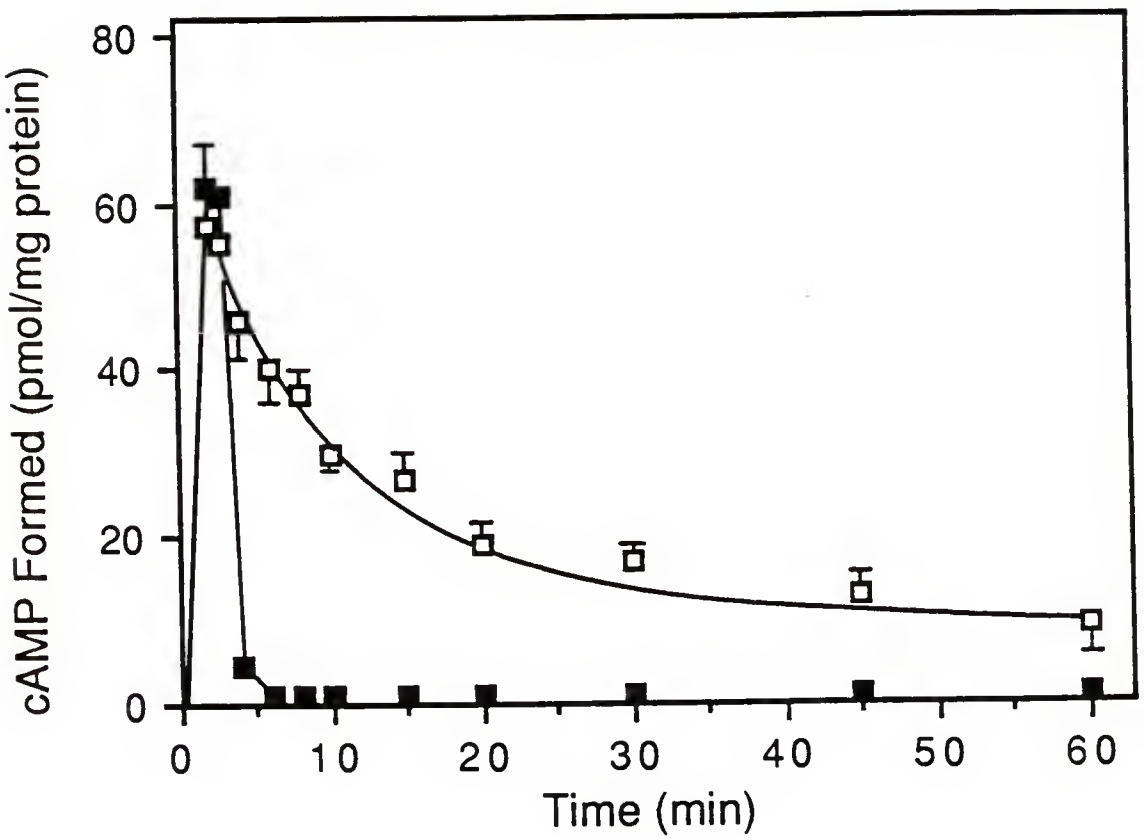

Figure 3-4. Time course of cAMP accumulation in intact DDT cells by Iso and Carbo-Br after addition of propranolol. Cyclic AMP accumulation was determined by incubating intact cells (0.36 mg) in HBSS buffer containing 10 µM Iso (closed squares) or 1 µM Carbo-Br (open squares) plus 20 µM propranolol added to all tubes after 3 min of incubation at 36°C. Determination of cAMP was performed as described under "Methods". Each data point was the mean of 3-4 determinations ± S.E. Basal cAMP formation has been subtracted from stimulated values.

increase (6-fold above basal) in cellular cAMP content followed by a relatively slow reduction over the next 57 min. When 20 μM propranolol was added to the system at the 3 min time point (Figure 3-4), there was an immediate drop in Iso-induced cAMP accumulation to basal levels. This was not the case, however, in the Carbo-Br-induced system. When propranolol was added after 3 min of incubation with Carbo-Br, the loss of cAMP accumulation over the next 57 min was similar to that observed in the absence of propranolol. This suggested that Carbo-Br had already bound to the receptors in a tight and/or irreversible manner. Phosphodiesterase activity was also measured after a time course of Carbo-Br and Iso pretreatment (Table 3-1). There was no difference in total phosphodiesterase activity over time with either pretreatment group as compared to control values.

Effects of Carbo-Br pretreatment on membrane adenylate cyclase activity. Table 3-2 showed the ability of Carbo-Br to stimulate cAMP production in DDT cell membranes under "Basal" cyclase conditions after intact cell pretreatment with Carbo-Br for 3, 30, and 60 min. The membranes from those cells which were pretreated with Carbo-Br (1 μM) and washed exhibited a 2.5-fold higher basal activity than the controls. In fact, the basal activity in membranes from 3 min Carbo-Br pretreated cells was as great as the Carbo-Br stimulated activity in membranes from control cells. There did appear to be a desensitization of this effect as the

Table 3-1. Effect of Carbo-Br and Iso pretreatment on phosphodiesterase activity in DDT cells

Pretreatment time (min)	[^{3}H]Adenosine formed (pmol/min/mg protein)	
	Carbo-Br	Iso
10	699.8 ± 80.5	326.8 ± 40.6
20	633.2 ± 97.0	558.2 ± 129.0
30	654.3 ± 67.8	510.8 ± 140.0
40	607.8 ± 43.0	492.2 ± 162.0
50	614.4 ± 26.9	533.5 ± 83.2
60	638.3 ± 10.6	542.7 ± 133.0
control	658.9 ± 26.0	517.2 ± 82.7

Intact cells (2.4 mg) were incubated for various times in HBSS buffer pH 7.4 containing 1 μM Carbo-Br or 10 μM Iso at 36°C. At the end of the incubations, cells were sedimented by centrifugation at 1000xg and resuspension in 0.9 ml of40 mM Tris-HCl. Cells were then homogenized, and the whole cell homogenate (0.3 mg/tube) assayed for phosphodiesterase activity as described in "Methods".

Each value is the mean ± SE, n=3.

Table 3-2. Stimulation of adenylate cyclase activity in DDT cell membranes after pretreatment of intact cells with Carbo-Br

Cellular Preincubation Conditions	Membrane cAMP production (pmol/10 min/mg protein) Basal	Carbo-Br (1 μM)	Carbo-Br plus Propranolol (20μM)
Control (3 min)	37.3 ± 4.4	115.3 ± 24.0	43.8 ± 6.3
Carbo-Br (3 min)	92.6 ± 9.6[a]	104 ± 11.0	90.0 ± 10.2[c]
Control (30 min)	40.0 ± 3	------	------
Carbo-Br (30 min)	74.4 ± 4.1[a]	------	------
Control (60 min)	38.1 ± 2.8	------	------
Carbo-Br (60 min)	53.2 ± 7.1[b]	------	------

Intact cells (1.5 mg) were incubated for various times in HBSS buffer at pH 7.4 in the presence or absence of 1 μM Carbo-Br. At the end of the incubations, the cells were washed four times by centrifugation at 1,000xg and resuspension. After the final wash, cells were resuspended in 50 mM Tris-HCl pH 7.4, homogenized, and the membranes sedimented by centrifugation at 35,000xg for 10 min. DDT cell membranes (65 μg/tube) were assayed for adenylate cyclase activity as described in "Methods", and the results were listed under the appropriate assay condition heading.

[a] Significantly different from the respective control, $p \leq 0.005$;
[b] Significantly different from the respective control, $p \leq 0.05$;
[c] Significantly different from the respective control, $p \leq 0.01$, as determined by unpaired student's t-test.
All values were means ± S.E., n=3-4.

basal activity declined in membranes from Carbo-Br treated cells over an hr, from 2.5-fold at 3 min to 1.4-fold stimulation at 60 min.

It was important to notice also that propranolol blocked Carbo-Br stimulated cAMP production in control membranes when the two were added together, but had no effect on cAMP production in membranes from cells that were pretreated with Carbo-Br (Table 3-2). This was consistent with our findings in reticulocytes and whole cells that Carbo-Br had bound tightly and/or irreversibly by at least 3 min, yet still stimulated cAMP production.

Effects of Carbo-Br and Iso pretreatment on DDT cell beta-adrenoreceptors. Receptor content was assayed in two ways: whole cell homogenates were assayed using [^{125}I]CYP, and intact cells were assayed using the hydrophilic beta-antagonist [^{3}H]CGP which measured only those receptors found on the cell surface (Hertel *et al.*, 1983; Wilkinson & Wilkinson, 1985). Figure 3-5 was a representative Scatchard plot of [^{125}I]CYP binding in whole cell homogenates after a 3 min pretreatment of intact cells with 1 μM Carbo-Br, 10 μM Iso or buffer alone followed by cell washing. The slopes of the lines were parallel, indicating that all of the unbound drugs have washed out (K_D values: control, 22 ± 4; Iso-treated, 16 ± 3; Carbo-Br-treated, 29± 8 pM, n=4-7). There was a 50% loss of binding sites after only a 3 min pretreatment with Carbo-Br (B_{max} values: control, 58 ± 3, Iso-treated, 57 ± 6; Carbo-Br-treated, 34 ± 5 fmol/mg

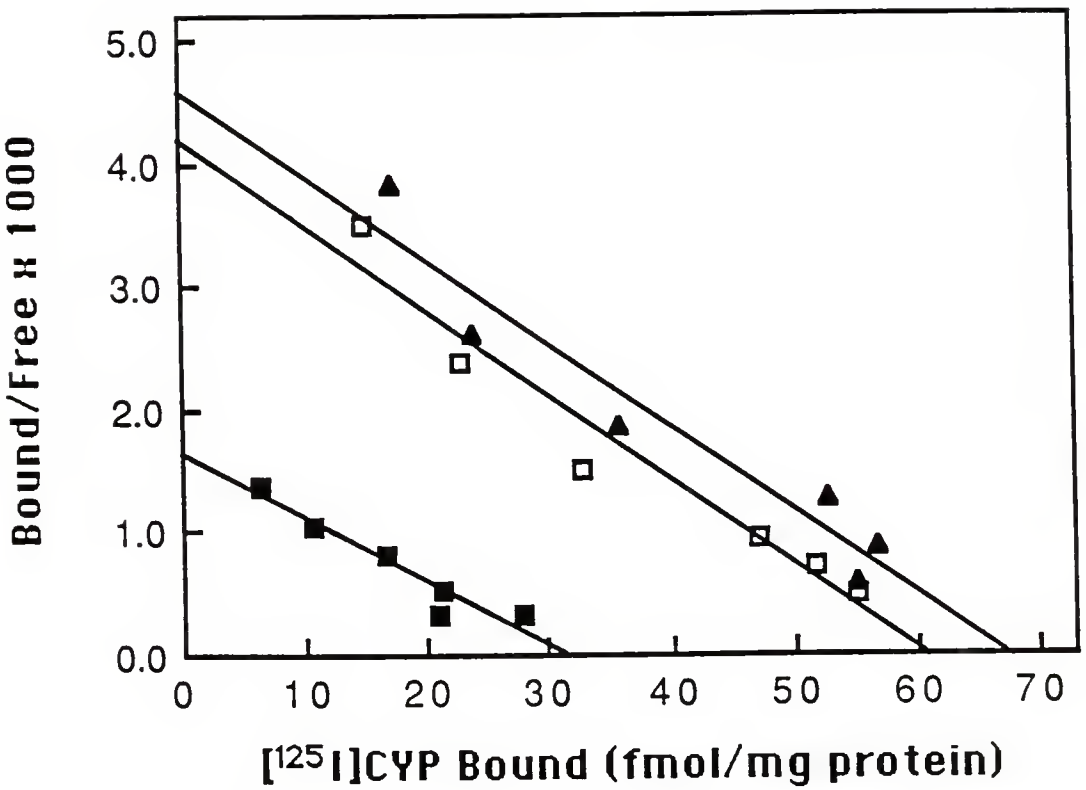

Figure 3-5. Scatchard plot of specific [^{125}I]CYP binding to whole DDT cell homogenates after intact cell treatment with Iso and Carbo-Br. Intact cells (3 mg) were incubated in HBSS buffer containing 10 µM Iso (triangles), 1 µM Carbo-Br (closed squares) or buffer alone (open squares) at 36°C for 3 min. Samples were removed, intact cells washed five times with ice-cold HBSS buffer, resuspended in 50 mM Tris containing 5 mM $MgCl_2$, and homogenized. The homogenates were assayed with 3 to 100 pM [^{125}I]CYP as described under "Methods". The data were plotted as the ratio of the amount of specifically bound ligand (pmol/mg protein) to free ligand (pmol/l) versus the amount of specifically bound ligand/mg protein. Data points were the mean of triplicate determinations and were representative of three experiments.

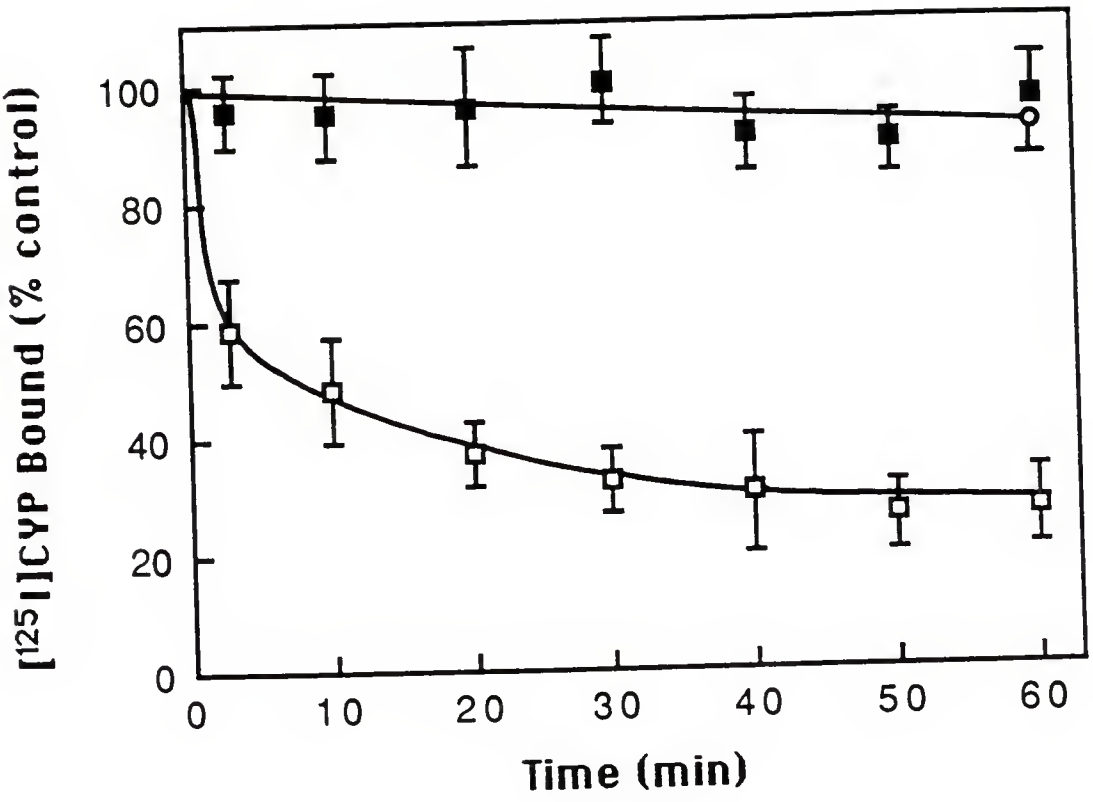

Figure 3-6. Time course of specific [^{125}I]CYP binding loss in whole DDT cell homogenates after intact cell treatment by Iso and Carbo-Br. Intact cells (3 mg) were incubated in HBSS buffer containing 10 µM Iso (closed squares), 1 µM Carbo-Br (open squares), or buffer alone (circles) at 36°C. At the times indicated, samples were removed, intact cells washed five times with ice-cold HBSS buffer, resuspended in 50 mM Tris-HCl containing 5 mM $MgCl_2$ and homogenized. The homogenates were assayed with 75 pM [^{125}I]CYP as described under "Methods". Data points were the mean of four determinations. The control [^{125}I]CYP values were 48 ± 4 fmol/mg protein.

protein, n=4-7). Similar results were obtained with [^{3}H]CGP (results not shown). There was no loss of binding sites after a 3 min pretreatment with Iso. Figure 3-6 presented a time course of the loss of specific binding sites with Carbo-Br, as measured with [^{125}I]CYP. There was a 42% loss of sites by 3 min with Carbo-Br that continued gradually, so that by 60 min there was a 70% loss of binding sites. Comparison with Iso-treated cells indicated that while the response may have been desensitized, there was no loss of binding sites as measured by [^{125}I]CYP binding in whole cell homogenates. Figure 3-7 demonstrated the loss of binding sites from the intact cell surface with both Iso and Carbo-Br pretreatment as measured by [^{3}H]CGP. After pretreatment with 10 μM Iso there was a 21% decrease in binding sites at 3 min that reached a 77% loss by 60 min. Carbo-Br showed a drop of 42, 76, and 82% over 3, 20, and 60 min, respectively. Figure 3-8 showed the recovery of specific [^{3}H]CGP binding sites after 1 hr of Carbo-Br or Iso pretreatment followed by a 24 hr incubation after the washout of unbound agonists. After the 1 hr pretreatment there was an 80 and 87% loss of binding sites with Iso and Carbo-Br, respectively. After 24 hr, Iso treated cells showed only a 27% decrease from controls, while Carbo-Br treated cells showed an 80% drop in binding sites.

Effects of Carbo-Br pretreatment on C6 cell membrane beta-adrenoreceptors. Since reticulocytes and DDT cells contained only receptors of the beta$_2$-subtype, it was of

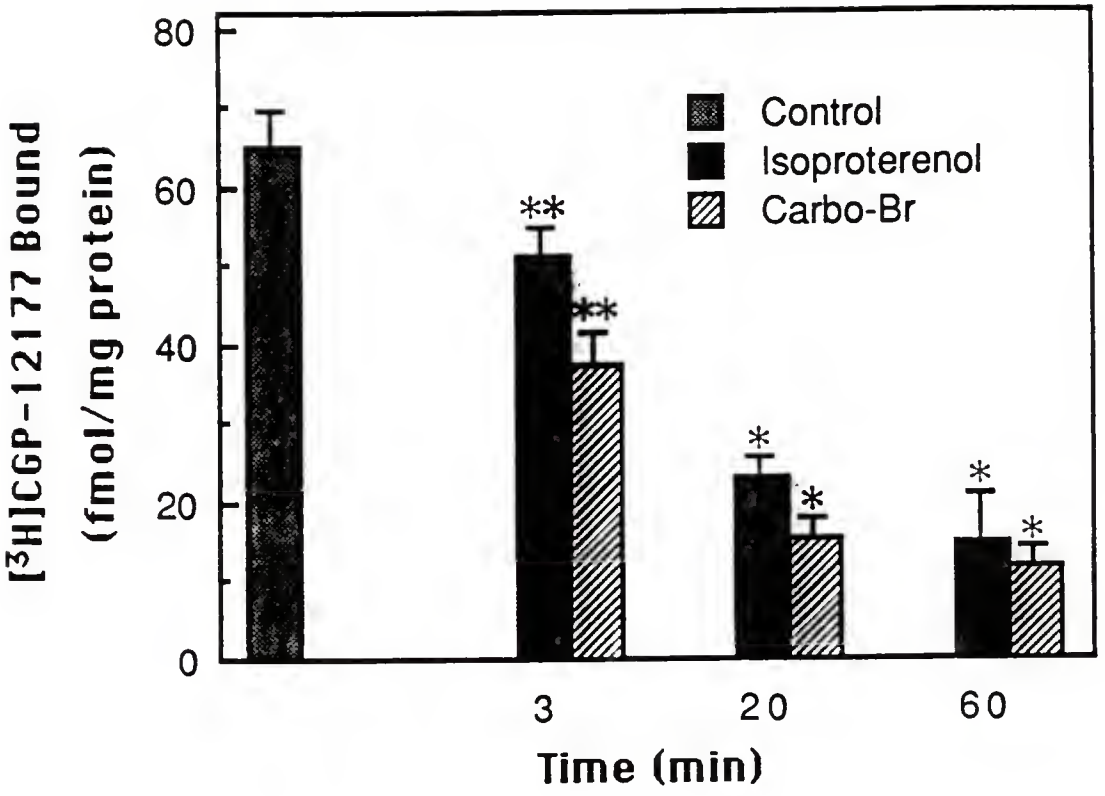

Figure 3-7. Time course of specific [^{3}H]CGP binding loss by Iso and Carbo-Br in intact DDT cells. Intact cells (6 mg) were incubated in HBSS buffer containing 10 µM Iso or 1 µM Carbo-Br at 36°C. At the times indicated, samples were removed to ice, intact cells washed five times with ice-cold HBSS buffer, resuspended in 4°C HBSS and assayed with 0.75 nM [^{3}H]CGP as described under "Methods". Data points were the mean of three determinations (significantly different from control, $^*p \leq 0.005$; $^{**}p \leq 0.05$). The control [^{3}H]CGP binding values ranged from 60.2 to 69.6 fmol/mg protein.

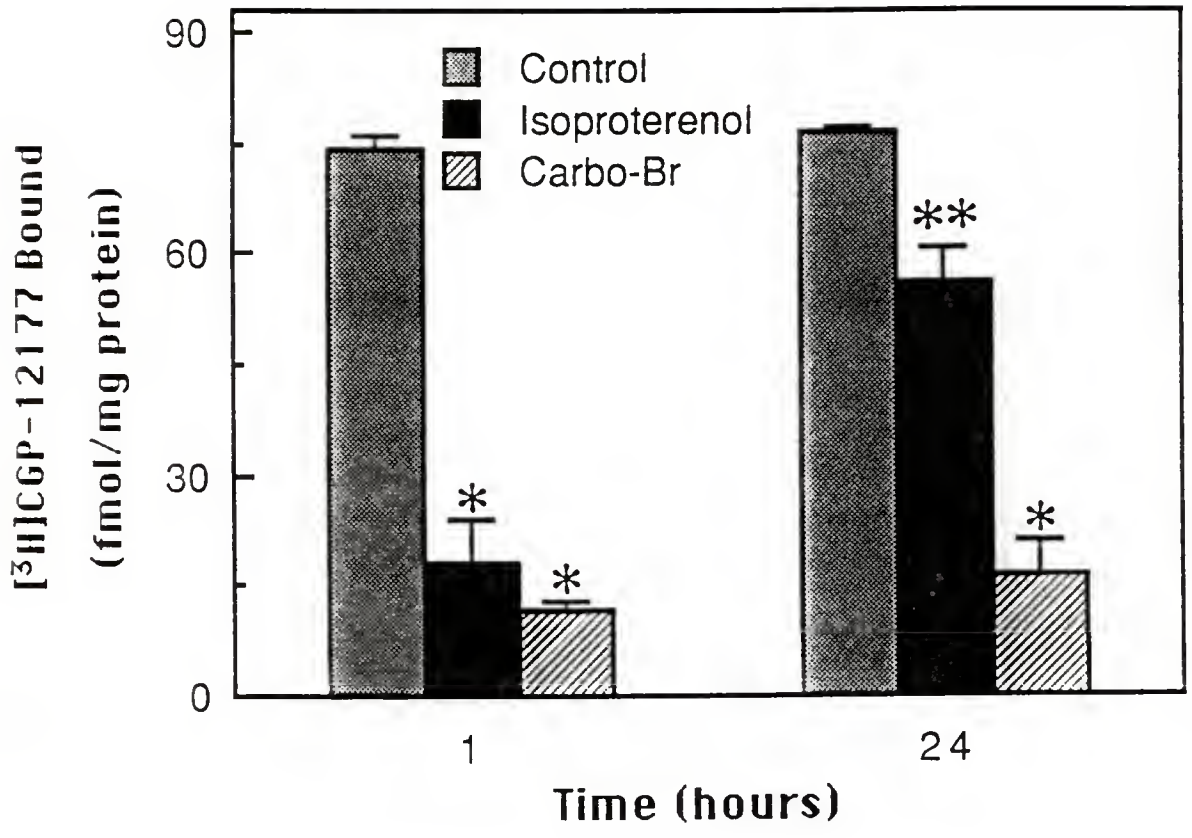

Figure 3-8. Loss of specific [^{3}H]CGP binding in intact DDT cells immediately or 24 hr after a 60 min treatment with Iso or Carbo-Br. DDT cells plated on 143 cm^2 plates were incubated in HBSS buffer containing 10 µM Iso or 1 µM Carbo-Br for 60 min at 36°C. At that time, the plates were removed to 4°C and washed five times with ice-cold HBSS buffer. One-half the cells from each treatment group were given DMEM plus 5% FCS and removed to the cell incubator. The remaining cells were lifted with ice-cold HBSS containing 1 mM EGTA, resuspended in 4°C HBSS and assayed with 0.75 nM [^{3}H]CGP as described under "Methods". Twenty-four hr later the rest of the cells were lifted and assayed as the former. Data points were the mean of three determinations (significantly different from control; $^*p \leq 0.0005$, $^{**}p \leq 0.025$). The control [^{3}H]CGP binding values were 74.3 ± 1.63 fmol/mg protein for 1 hr and 76.7 ± 0.76 for 24 hr (mean + S.E.).

Table 3-3. Loss of binding sites in two C6 clones after Carbo-Br pretreatment

	B_{max} (fmol/mg protein)	
Cell line	Control	Carbo-Br
C6	33.3 ± 0.1	25.2 ± 1.5[a]
C62B	16.3 ± 1.4	13.6 ± 1.6

Cell membranes (4.0 mg) were preincubated in the presence or absence of 1 μM Carbo-Br for 15 min at 36°C in 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$ and 100 μM Gpp(NH)p. At the end of that time, membranes were washed 5 times by centrifugation at 35,000xg and resuspension. Membranes (25 μg/tube) were assayed with 3-100 pM [^{125}I]CYP as described in "Methods".

[a] Significantly different from control, $p \leq 0.025$, as determined by unpaired student's t-test.

All values given as means ± S.E., n=3.

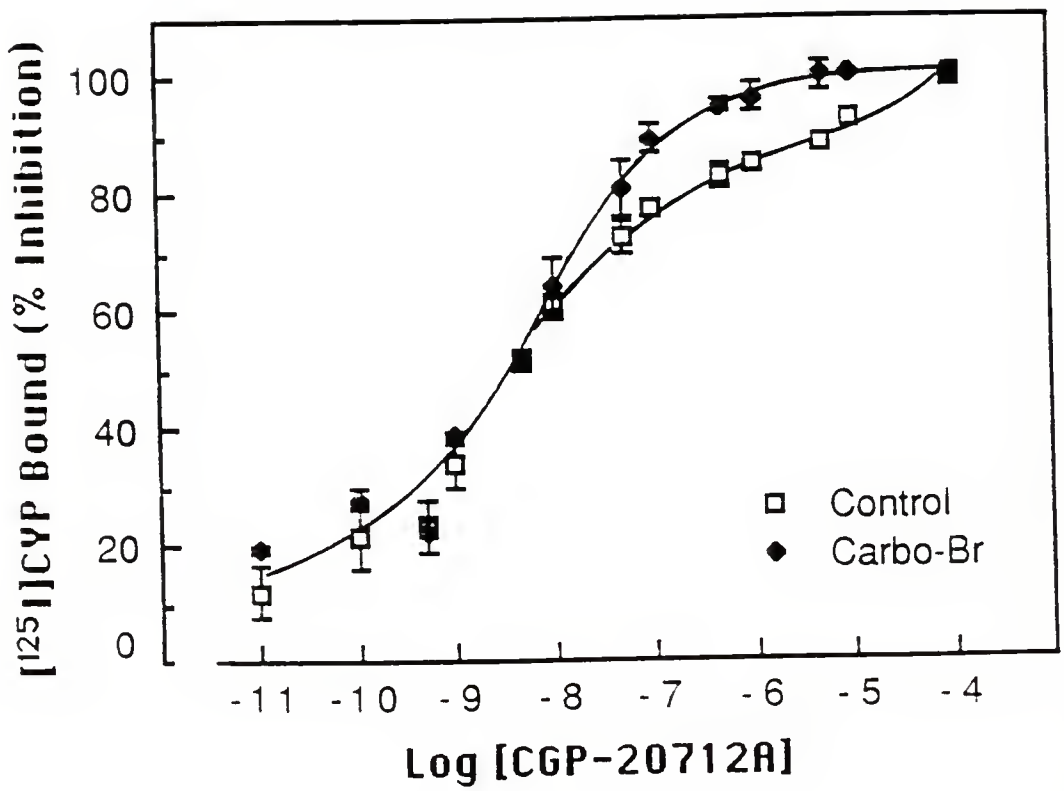

Figure 3-9. Inhibition of specific [^{125}I]CYP binding in C6 cell membranes by CGP-20712A after Carbo-Br treatment. Membranes (4.0 mg)were incubated with buffer at pH 7.4 in the presence or absence of 1 µM Carbo-Br for 15 min at 36°C. The membranes were washed five times with ice-cold buffer and assayed with 30 pM [^{125}I]CYP, 100 µM Gpp(NH)p, and the indicated concentrations of CGP-20712A for 45 min at 36°C. The curves were representative of three experiments. Specific [^{125}I]CYP binding totals were 18.6 and 13.8 fmol/mg protein for control and Carbo-Br treated membranes, respectively.

interest to determine the effects of Carbo-Br binding on $beta_1$-adrenoreceptors. Table 3-3 compared the loss of binding sites in two other cell lines, C6 and its subclone C62B, after Carbo-Br pretreatment. The C62B cell membranes, which appeared to contain only $beta_1$-adrenoreceptors, showed no loss of binding sites after Carbo-Br pretreatment. Membranes from C6 cells, on the other hand, exhibited a 25% loss in binding sites after Carbo-Br pretreatment. The IC_{50} values for Carbo-Br inhibition of specific [^{125}I]CYP binding in C6 and C62B membranes in the presence of 100 µM Gpp(NH)p were the same, 40 nM ± 5 (n=2-5). Figure 3-9 showed the ability of CGP-20712A to inhibit specific [^{125}I]CYP binding in membranes from C6 cells. In control membranes the inhibition curve was biphasic. Using a multisite analysis, the data best fit a 2-site model with 82% of the sites showing high affinity for CGP-20712A ($beta_1$ subtype) and 18% of the sites with low affinity ($beta_2$ subtype). After pretreatment of the C6 membranes with Carbo-Br (1 µM), the CGP-20712A competition data best fit a single binding site model with only high affinity for the antagonist. There may have been a small number of low affinity sites present, but they were within the error of the assay. The data indicated that Carbo-Br mainly bound in a tight and/or irreversible manner to the low affinity ($beta_2$ subtype) CGP-20712A binding sites.

Effects of Carbo-Br on alpha-adrenoreceptor binding.

The fact that the DDT cells contained $alpha_1$- (Cornett & Norris, 1982) as well as $beta_2$-adrenoreceptors allowed for

the examination of Carbo-Br binding to alpha$_1$-adrenoreceptors. Carbo-Br bound to the beta$_2$-adrenoreceptors of DDT cell membranes with an IC_{50} value of 9 nM. In contrast, Carbo-Br displaced [^{3}H]Prazosin binding with an IC_{50} value of 16,000 nM for the alpha$_1$-adrenoreceptor. Therefore, there was a large difference in potencies for Carbo-Br for the alpha$_1$- and beta$_2$-adrenoreceptors.

Discussion

We have shown that Carbo-Br produced sustained activation of adenylate cyclase in reticulocyte membranes after binding tightly and/or irreversibly to the receptor. We have further investigated the effects of Carbo-Br using an intact cell system. Similar to the reticulocyte studies, Carbo-Br was more potent than Iso at inhibiting specific [^{125}I]CYP binding and stimulating cAMP production in DDT cell membranes and intact cells. It also exhibited the same intrinsic activity as Iso, indicating that Carbo-Br was a full agonist in this system as well as in the reticulocyte membrane system.

That Carbo-Br produced desensitization of beta-adrenoreceptor-coupled adenylate cyclase activity was shown in two ways. First, cAMP levels in intact cells dropped to near basal levels within 57 min of being stimulated 6-fold by 1 μM Carbo-Br (almost identical to the pattern elicited by 10 μM Iso). The decline in cAMP levels cannot be attributed to differential effects of Carbo-Br on phosphodiesterase

activity, as neither Iso nor Carbo-Br affected the activity of that enzyme. Second, studies of cAMP production in membranes produced from intact cells which had been pretreated with Carbo-Br also appeared to undergo a desensitization. Membranes from cells preincubated for 30 and 60 min with 1 μM Carbo-Br only produced 80 and 57%, respectively, as much cAMP as the 3 min treated ones. Interestingly, the desensitization observed in the membrane assays appeared to be slower than that observed in the intact cells. This discrepancy may be a reflection of the intrinsic differences in assay procedure, the presence (membrane assays) or absence (intact cell assays) of a phosphodiesterase inhibitor, or an active cellular extrusion mechanism to remove cAMP from intact cells. Nonetheless, both experimental approaches indicated that Carbo-Br was producing a desensitization. A desensitization phenomenon in DDT cells has been reported by others (Strasser *et al.*, 1986; Cowlen & Toews, 1987; Toews *et al.*, 1987).

That Carbo-Br was binding either quasi-irreversibly or covalently to the beta-adrenoreceptors of DDT cells was supported by both binding and cAMP stimulation studies. The inability of propranolol to reverse Carbo-Br-induced cAMP accumulation in intact cells or cell membranes after 3 min implied that Carbo-Br had bound tightly and/or irreversibly by that time. A 40-50% loss of binding sites was detected after only a 3 min pretreatment of intact cells with Carbo-Br with no change in the K_D of [^{125}I]CYP or [^{3}H]CGP for the

remaining receptors. By measuring beta-adrenoreceptor binding in two ways, we showed the pattern of Iso-induced receptor redistribution and compared it to Carbo-Br-induced receptor loss. Using whole cell homogenates allowed any internalized receptors to be accessible to [^{125}I]CYP, and measured all structurally intact beta-adrenoreceptors (Motulsky *et al.*, 1986). Because [^{3}H]CGP is hydrophilic, it has be used to measure only those beta-adrenoreceptors found on the cell surface (Hertel *et al.*, 1983; Wilkinson & Wilkinson, 1985). It was shown that acute Iso treatment reduced [^{3}H]CGP binding to intact cells with no change in [^{125}I]CYP binding to cell homogenates indicating an agonist-induced receptor redistribution, not receptor loss. In contrast, there was a receptor loss during Carbo-Br pretreatment that was similar when measured with either [^{125}I]CYP or [^{3}H]CGP. At this point it was unclear whether Carbo-Br-bound receptors were internalized or not. What was clear, however, was that after 24 hr, almost all of the Iso-internalized receptors had reappeared on the cell surface. While the cellular location of Carbo-Br-bound receptors was not clear, it appeared that Carbo-Br had not dissoci-ated from them, even after 24 hr. The other possibility was that the Carbo-Br-bound receptors were signalled for degradation. Current evidence has indicated that acute agonist treatment uncoupled the receptor from N_S. Agonist affinity for the receptor in the uncoupled state was low and suggested that freely reversible agonists would dissociate during or before receptor

internalization (Hertel & Perkins, 1984). Since Carbo-Br was tightly or irreversibly bound to the receptor, it seems probable that if internalization occurred, Carbo-Br would be still attached to the receptor and might have increased the rate of receptor degradation as has been observed with chronic agonist treatment (Hertel & Perkins, 1984). Once Carbo-Br is radiolabelled, it might be possible to learn what was happening to the receptor. If indeed it was being internalized, a radiolabelled irreversible agonist might give us the most accurate picture yet of agonist-induced receptor processing. There has been little doubt that agonist-induced internalization and down-regulation set in motion a different chain of events than normal basal receptor turnover. For example, agonist-induced internalization appeared to increase the rate of receptor clearance from the cell surface and degradation (Mahan *et al.*, 1987). It seemed logical, therefore, that an irreversible radiolabelled agonist might provide different information than a radiolabelled irreversible antagonist. Together, they might be able to provide a more detailed picture of basal and agonist-induced receptor turnover.

Since all of our studies to this point had included only systems composed of pure $beta_2$-adrenoreceptor populations, we were curious as to whether Carbo-Br would show the same affinity and irreversible properties for $beta_1$-adrenoreceptors. The C6 glioma line contained 80% $beta_1$- and 20% $beta_2$-receptors (Homburger *et al.*, 1981; Harden & McCarthy,

1982), C62B membranes consisted primarily of $beta_1$-adrenoreceptors. Pretreatment of membranes from both cell types with Carbo-Br at a concentration that occupied greater than 90% of the receptors resulted in a 25% loss of binding sites from C6 membranes with no loss of sites in C62B membranes. It was of interest to determine if the 25% loss of binding sites in C6 membranes corresponded to the 20% $beta_2$-receptor population there. Using the $beta_1$-selective antagonist CGP-20712A (Dooley *et al.*, 1986; Brodde, 1987), we demonstrated a change in the $beta_1$ to $beta_2$ ratio after Carbo-Br pretreatment of C6 membranes. It appeared that 1 µM Carbo-Br bound quasi-irreversibly to the $beta_2$-adrenoreceptors on those membranes, with only one receptor population remaining ($beta_1$). If Carbo-Br was only binding tightly to the $beta_2$-adrenoreceptor, it argues strongly for important structural differences in the ligand binding sites between the two subtypes. If Carbo-Br could be radiolabelled, it could be used to distinguish differences between the ligand binding sites of the two subtypes by using the method recently described by Dohlman *et al.* (1988).

Finally, it appeared that Carbo-Br was able to compete with [^{3}H]Prazosin for $alpha_1$-adrenoreceptor binding sites. While the drug was much more selective for beta- than $alpha_1$-receptors, the IC_{50} of Carbo-Br for inhibiting [^{3}H]Prazosin binding was comparable to both the alpha-antagonist yohimbine (4.6 µM) and the agonist epinephrine (27.8 µM) as measured in DDT cells (Toews, 1987). Whether or not Carbo-Br was an

alpha agonist or antagonist, and if it bound in an irreversible manner to the alpha-adrenoreceptor would require further study.

CHAPTER 4
WHOLE ANIMAL STUDIES

Introduction

As shown in the previous chapters, Carbo-Br was a potent, stable, beta-adrenoreceptor agonist that seemed to bind irreversibly to membranes and intact cells. Though Carbo-Br produced a sustained activation of adenylate cyclase in reticulocyte membranes, it appeared to trigger a desensitization response in intact cells, similar to other freely reversible beta-agonists. Moreover, the extremely tight and/or irreversible binding of Carbo-Br was specific for the $beta_2$-receptor subtype. Because of the complex interaction of Carbo-Br with the beta-adrenoreceptor, it was important to characterize its effects when administered to animals. Most tissues *in vivo* contained beta-adrenoreceptors with the subtype ratio varying considerably. Furthermore, beta-adrenoreceptors mediated a variety of important physiological responses, and both beta-agonists and antagonists have salutary effects in the treatment of a number of disorders. In the long-term, the development of beta-adrenoreceptor compounds which act differently than those of the catecholamines may provide further insight on the regulation and role of the beta-adrenoreceptor in health and disease.

Therefore, the interaction of Carbo-Br with beta-adrenoreceptors from several tissues of the rat were partially characterized.

Experimental Procedures

Source of Materials

The radiolabelled compounds L-[^{14}C]ornithine (52-59 Ci/mmol) and (-)-[^{3}H]dihydroalprenolol ([^{3}H]DHA; 48.6-60.0 Ci/mmol), Econofluor and Protosol were purchased from New England Nuclear, Boston, MA, USA. Dithiothreitol and pyridoxyl phosphate were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Methods

Drug treatments. Stock solutions of Carbo-Br (1 mM) were prepared fresh monthly by dissolving the compound in ethanol. Dilutions from the stock solution were made with distilled water only. Male Sprague-Dawley rats (200 g) were injected i.p. or s.c. with 0.5, 2.0, 5.0, or 10.0 mg/kg of the drug. Control rats were injected i.p. or s.c. with the ethanol-water vehicle only.

Membrane preparation. Rats were decapitated and the hearts, lung lobes, spleens, submaxillary glands, and brain cortices removed to ice-cold saline. Atria, valves and fat were dissected from the heart ventricles. The tissues were rinsed, blotted, weighed, and all tissues but the atria,

ventricles and brain were frozen in liquid nitrogen for assays performed no longer than one week later.

Tissues were cut into small pieces and homogenized in 15 volumes (w/v) of ice-cold 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$ and 0.32 M sucrose with a Tekmar SDT-182EN homogenizer at setting 5 for 15 sec. For atria, the homogenate was placed on ice for 1 min and homogenized again. The suspensions were diluted with 20 ml of homogenization buffer and centrifuged at 48,000xg for 10 min in a Sorvall RC-5B centrifuge. The supernatant was discarded and the pellet resuspended (setting 3, 10 sec) in 20 ml of ice-cold 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$, passed through a tea sieve (30 mesh nylon sieve for heart) to remove large pieces of connective tissue, and centrifuged at 48,000xg for 10 min. The resulting pellets were washed twice more and resuspended in 1 volume of 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$ for assays.

Membrane pretreatments. In the isolated membrane studies, membrane protein (3.5 mg/ml, heart; 2.0 mg/ml, lung; 3.0 mg/ml, spleen; and 3.0 mg/ml, submaxillary gland) was incubated with 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$, Carbo-Br and with 100 μM Gpp(NH)p at 30°C for the times indicated. At the end of the incubation, suspensions were diluted with 30 ml cold buffer pH 7.4 and centrifuged at 48,000xg for 10 min. Pellets were resuspended and centrifuged three more times as above, and final pellets suspended in 3 ml buffer for assays.

Antagonist binding assays. Beta-adrenoreceptor concentration was determined by incubating membrane protein (0.07-0.08 mg, heart; 0.05 mg, cerebral cortex, spleen and submaxillary gland) in a total volume of 0.25 ml with 50 mM Tris-HCl buffer pH 7.4 containing 5 mM $MgCl_2$, 6.25-100 pM [^{125}I]CYP, and in the presence and absence of 1 μM (±)alprenolol for 60 min at 36°C. At the end of the incubation, each suspension was diluted with 3 ml of 50 mM Tris-HCl buffer at pH 7.4 (36°C) containing 5 mM $MgCl_2$, poured onto a Whatman GF/C glass fiber filter under reduced pressure. Filters were quickly washed with an additional 6 ml of buffer, placed in vials, and radioactivity determined. Specific binding was determined as previously described.

In some experiments, the ability of various concentrations of Iso and Carbo-Br to inhibit specific [^{125}I]CYP binding was performed. Assays were the same as above except the [^{125}I]CYP concentration was 30 pM and 0.1% sodium ascorbate was included to retard the oxidation of Iso. These competitive binding assays were performed in the presence or absence of 100 μM Gpp(NH)p. All binding assays were performed in triplicate.

Beta-adrenoreceptor content in lung membranes was determined by incubating membrane protein (0.25 mg/tube) in 50 mM Tris-HCl pH 7.4 containing 5 mM $MgCl_2$, 0.15-5.0 nM [^{3}H]DHA, and in the presence and absence of 1 μM (±)alprenolol for 30 min at 36°C. At the end of that time, 4 ml of 50 mM Tris-HCl containing 5 mM $MgCl_2$ at 4°C was added to each tube, and the

suspensions were poured onto GF/C glass fiber filters under reduced pressure. Filters were quickly washed with an additional 8 ml of buffer, placed in scintillation vials with 7 ml Liquiscint and radioactivity determined. Specific binding was determined as previously described. The use of [^{3}H]DHA instead of [^{125}I]CYP was necessitated by the inclusion of incompletely homogenized tissue in a given assay tube. The large disparity in the data observed with the use of [^{125}I]CYP (with its high specific activity) was reduced with [^{3}H]DHA.

Beating atrial preparations. Rats were killed by decapitation and the hearts quickly removed. The atria containing the sinus node (S-A) were immersed in modified Tyrode's solution (NaCl, 136.9 mM; KCl, 5.36 mM; NaH_2PO_4, 0.33 mM; $MgCl_2$, 2.3 mM; $CaCl_2$, 2.0 mM; HEPES, 5.0 mM; D-glucose, 5.0 mM; sodium ascorbate, 50 μM; bubbled with 100% O_2, pH 7.4). Tissue containing the S-A pacemaker cells, adjacent segments of the crista terminals and atrial appendage, were dissected free and mounted (endocardial surface up) in a constant temperature perfusion bath (36°C). The preparation was superfused with modified Tyrode's solution and allowed to beat spontaneously. Changes in beating rate were measured by using standard microelectrode techniques to record transmembrane action potentials from the S-A-node-atrial preparation as described previously (Baker & Posner, 1986; Carpentier *et al.*, 1984).

The spontaneous beating rate was allowed to stabilize at least 1 hr, at which time the action potential configuration was observed and, if not normal, the preparation not used. At the end of the equilibration period, cumulative dose response data were obtained by superfusing the drug for 5 min, at which time the beating rate was recorded. For time course studies, the preparations were superfused with 0.1 μM Iso or Carbo-Br for 20 min. After that time, the tissues were immediately superfused with 50 nM nadolol until the beating rates returned to basal levels and stabilized.

Ornithine decarboxylase (ODC) activity. Rats were decapitated 3 and 24 hr after a 0.5 ml s.c. injection containing 0.1 or 5.0 mg/kg of either Iso or Carbo-Br. Basal ODC activity was determined 3 or 24 hr after a s.c. injection of saline as described by Nelson *et al.* (1987). Briefly, the hearts and lungs were rapidly removed after decapitation and rinsed in ice-cold saline. The atria were dissected free of the heart, and the ventricles and lungs were each homogenized in 10 volumes of a 25 mM Tris-HCl buffer pH 7.2 containing 0.5 mM dithiothreitol and 50 μM pyridoxyl phosphate for 20 sec at setting 5 with Tekmar SDT-100EN. The homogenates were centrifuged at 40,000xg for 15 min, and the supernatant fraction saved for the assay. Supernatant (200 μl) was added to 50 μl of homogenization buffer containing 60 μM [^{14}C]ornithine in an air-tight 10-ml side arm flask (Kontes, Vineland, NJ, USA) and incubated for 60 min at 36°C. The assay was stopped by adding 0.5 ml of 1 M citric acid to the

incubation mixture. Protosol (0.2 ml) was added to the center well of the flask by syringe to absorb the $^{14}CO_2$. The flasks were incubated for an additional 30 min at 36° C, after which the center wells were removed, placed in 10 ml Econofluor, and the radioactivity determined. Blanks were determined in the absence of protein.

Synaptosomal preparation. Male Sprague-Dawley rats were decapitated and the cortical tissues rapidly dissected. A crude synaptosomal (P_2) fraction was prepared essentially as described by Gray & Whittaker (1962). Briefly, the tissue was homogenized in 20 volumes of ice-cold 0.32 M sucrose using a Teflon homogenizer (10 strokes, 900 rpm). The homogenate was centrifuged at 1,000xg for 20 min, and the supernatant discarded. The pellet (P_2) was gently resuspended in oxygenated incubation buffer pH 7.4 containing 10 mM glucose, 20 mM HEPES, 145 mM NaCl, 4.5 mM KCl, 1.2 mM $MgCl_2$, and 1.5 mM $CaCl_2$ for assays.

[^{3}H] Norepinephrine uptake assay. Synaptosomal protein (0.2-0.3 mg) was suspended in a total volume of 1.0 ml containing oxygenated incubation buffer, 10 µM pargyline (to inhibit monoamine oxidase), 200 µM sodium ascorbate, and other drugs as indicated in the text. Synaptosomal protein (5 mg/ml) was incubated with oxygenated incubation buffer in the presence and absence of desired concentrations of Carbo-Br (10^{-9} M to 10^{-4} M) at 37°C for 30 min. At that time, 50 nM unlabelled L-NE plus 0.45 µCi of [^{3}H]NE was added to the assay tubes. Incubations were carried out at 37°C for 6 min,

after which time the suspensions were diluted with 4 ml of ice-cold incubation buffer. The suspensions were poured onto 0.45 μm Millipore filters under reduced pressure. The filters were washed with a further 8 ml of ice-cold buffer, placed in a scintillation vial with 8 ml of Liquiscint, and the radioactivity determined. Net NE uptake was calculated by subtacting the blank values obtained by the substitution of LiCl for NaCl. All assays were performed in triplicate.

Data analysis. Data was analyzed using a Student's t-test or analysis of variance as described in text.

Results

Binding of Carbo-Br to beta-adrenoreceptors *in vivo*.

Male Sprague-Dawley rats were injected i.p with various doses of Carbo-Br (0.5, 2.0, 5.0, or 10.0 mg/kg). Figure 4-1 showed the receptor loss in 5 tissues, 3 hours following the injection. The observation that no loss of receptors was seen in the cerebral cortex (and cerebellum, data not shown) suggested that this compound was not crossing the blood-brain-barrier. There was a dose-dependent reduction in binding sites in lung and spleen to a maximal loss of about 70-75% at 5.0 and 10.0 mg/kg. In contrast, the heart and submaxillary glands showed only a 15-25% loss of binding sites, even at high doses of Carbo-Br. Similar losses of receptor binding at the 5 mg/kg dose were observed when the drug was given s.c. (data not shown). No changes in the K_D values for [^{125}I]CYP or [^{3}H]DHA were observed using any

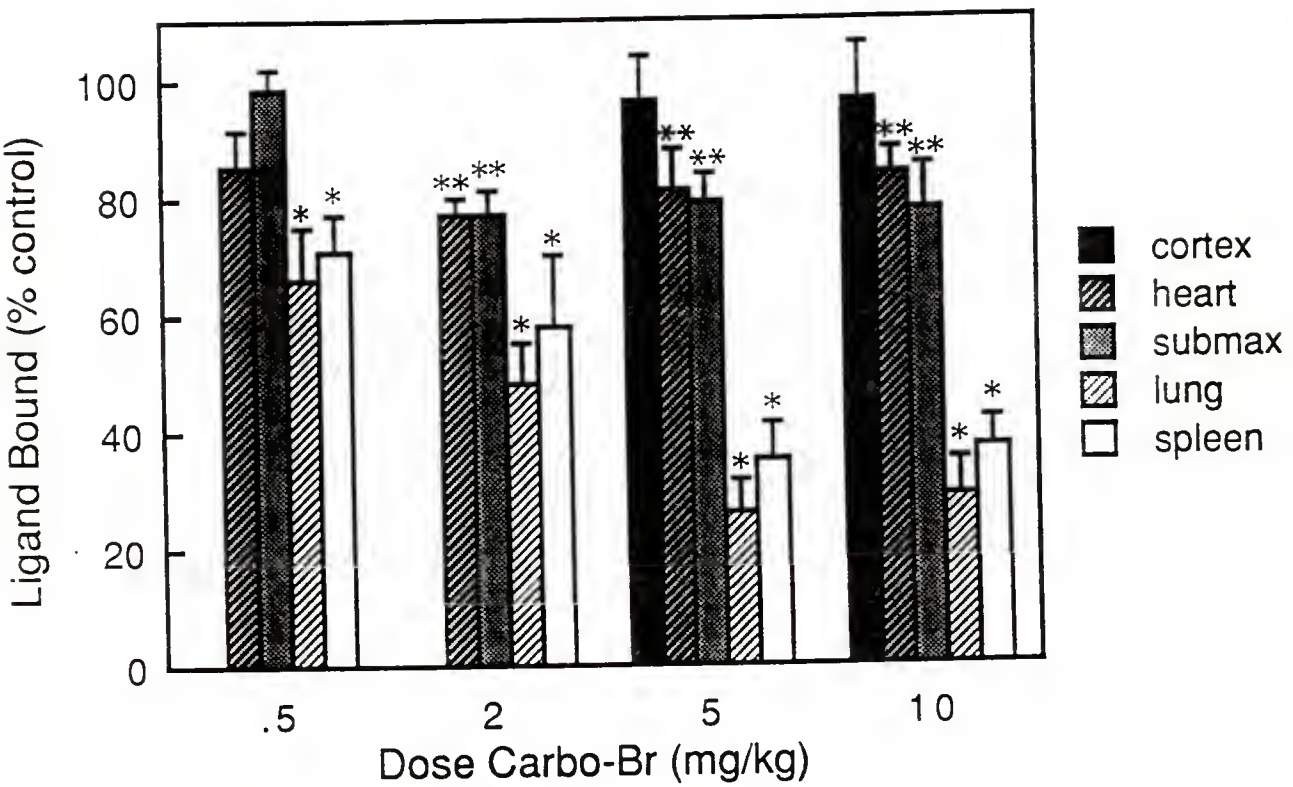

Figure 4-1. Effect of *in vivo* Carbo-Br treatment on beta-adrenoreceptors in 5 tissues of the rat. Male rats were injected i.p. with 0.5, 2, 5, or 10 mg/kg of Carbo-Br. After 3 hr, cerebral cortices, submaxillary glands, hearts, lungs, and spleens were removed. Membranes were prepared and assayed for beta-adrenoreceptor content with 3-100 pM [^{125}I]CYP or 0.15-5 nM [^{3}H]DHA as described under "Methods". Specific binding for each tissue was as follows: submaxillary gland, 98 ± 5.8; heart, 22.3 ± 1.0; lung, 658 ± 29; spleen, 53.2 ± 1.8; cerebral cortex, 119 ± 10 fmol/mg protein. The values for each group were the mean of 7-12 animals ± S.D (significantly different from control: $^{*}p \leq 0.0005$; $^{**}p \leq 0.05$).

of the tissues or at any dose of Carbo-Br as compared to control values (data not shown).

To test for a selective loss of beta-adrenoceptor subtype, the ability of the highly $beta_1$-selective antagonist, CGP-20712A, to inhibit subtype non-selective [^{125}I]CYP binding after animal treatment with Carbo-Br was examined. The results were shown in Figure 4-2. In control membranes, the ratio of $beta_1$ to $beta_2$ estimated from the plateau region was: lung, 22/78; heart, 75/25; spleen, 17/83; submaxillary gland, 82/18. Three hours after a 5 mg/kg injection of Carbo-Br, the ratio of $beta_1$ to $beta_2$ was increased to: 63/37, lung; 43/57, spleen; 95/5, submaxillary gland; and 90/10, heart. This seemed to indicate that the majority of receptor loss was at the $beta_2$-receptor, with little or no irreversible binding of Carbo-Br to the $beta_1$-receptor.

Binding of Carbo-Br to beta-adrenoceptors *in vitro*.

Table 4-1 showed the ability of Iso and Carbo-Br to inhibit specific radioligand binding to membranes from four rat tissues. Carbo-Br was found to be 4.6-fold more potent in the heart and submaxillary gland and 22 to 26-fold more potent in the lung and spleen than Iso at inhibiting specific ligand binding.

Figure 4-3 showed a representative Scatchard plot of [^{3}H]DHA binding to lung membranes after a preincubation with 0.5 μM Carbo-Br. Membranes were preincubated with the compound for 30 min at 30°C in the presence and absence of 100 μM Gpp(NH)p and washed four times. Compared to the

Figure 4-2. Inhibition of specific [^{125}I]CYP and [^{3}H]DHA binding in rat tissue membranes by CGP-20712A following *in vivo* Carbo-Br treatment. A) Heart (squares), spleen (circles); B) sumaxillary gland (squares), lung (circles). Male rats were injected i.p. with 5 mg/kg Carbo-Br (closed symbols) or vehicle (open symbols). After 3 hr, submaxillary glands, hearts, lungs, and spleens were removed. Membranes were prepared and assayed with 30 pM [^{125}I]CYP or 5 nM [^{3}H]DHA, and the indicated concentrations of CGP-20712A for 45 min at 36°C. At the end of the incubations, specific binding was determined as described under "Methods". Each point on the graph was the mean of four determinations assayed in triplicate. The control [^{125}I]CYP binding values were 48 ± 2, 14.5 ± 0.5, and 50 ± 2.6 fmol/mg protein for the submaxillary gland, heart, and spleen, respectively. The control [^{3}H]DHA binding value for the lung was 343 ± 28 fmol/mg protein. Specific [^{125}I]CYP binding values for Carbo-Br treated tissues were submaxillary gland, 37.5 ± 1.8; heart, 8.8 ± 0.25; and spleen, 6.2 ± 0.3 fmol/mg protein. Specific [^{3}H]DHA binding for the Carbo-Br treated lung tissue was 76.6 ± 6.3 fmol/mg protein. Each value was the mean of six to eight determinations.

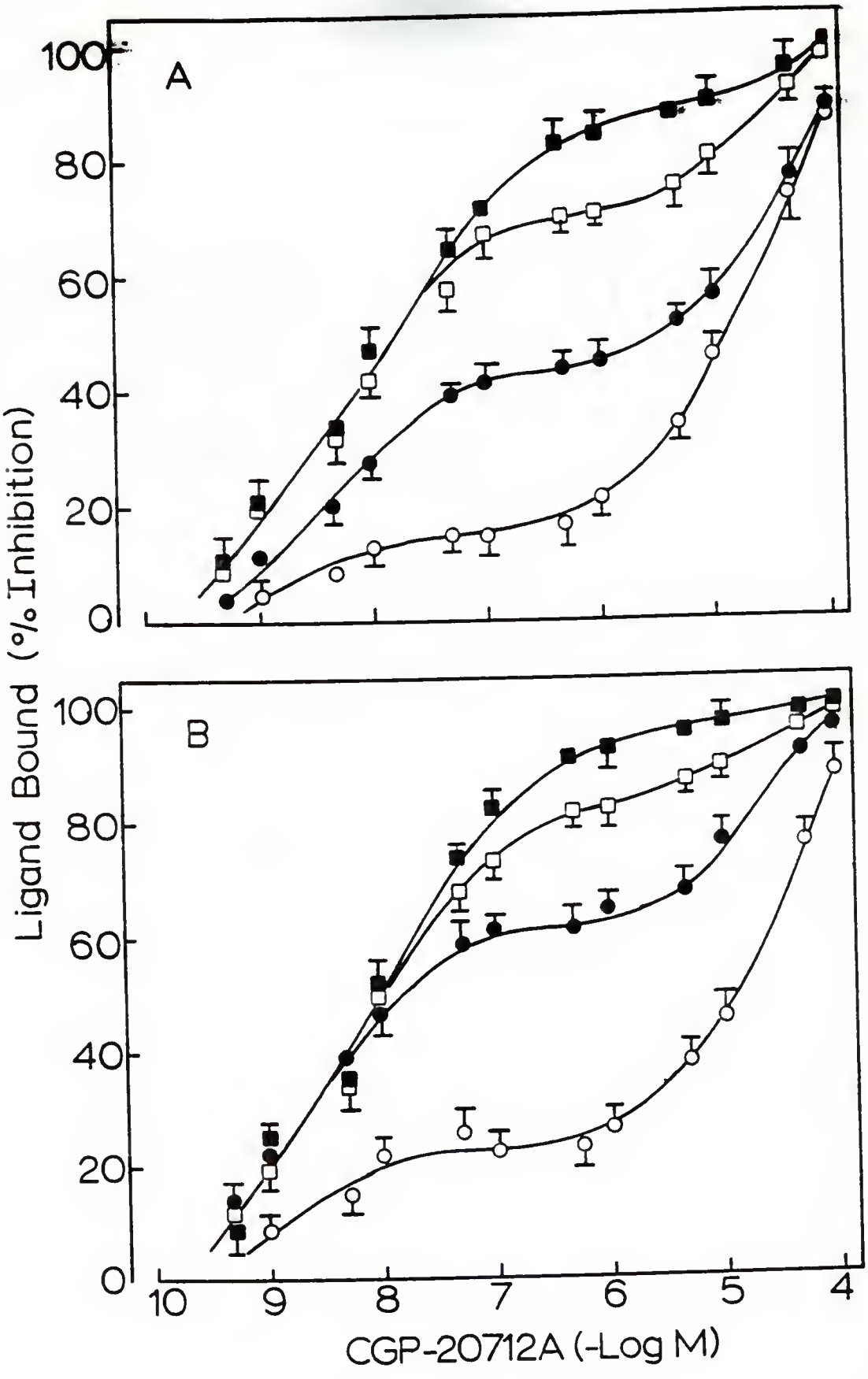
A
B
100
80
60
40
20
0
Ligand Bound (% Inhibition)
10
9
8
7
6
5
4
CGP-20712A (-Log M)

Table 4-1. Concentration of Iso and Carbo-Br that inhibit specific ligand binding to the beta-adrenoreceptors from several rat tissues by 50% (IC_{50})

Tissue	IC_{50} (nM)[a]	
	Iso	Carbo-Br
Heart	443 ± 55	95 ± 7[b]
Submaxillary gland	449 ± 41	96 ± 11[b]
Spleen	613 ± 23	23 ± 6[b]
Lung	543 ± 22	24 ± 5[b]

Membranes from each tissue were incubated with 50 mM Tris-HCl buffer at pH 7.4 containing 5 mM $MgCl_2$, various concentations of Iso or Carbo-Br, and 100 µM Gpp(NH)p for 45 min at 36°C. The incubations also included 30 pM [^{125}I]CYP for heart, submaxillary gland, and spleen assays, and 5 nM [^{3}H]DHA for lung assays. The total specific binding values were similar to those given as control values in Figure 4-2.

[a] Each value was the mean ± S.E., n= 4-8.
[b] Significantly different from Iso values, $p < 0.0005$ as determined with unpaired student's t-test.

control, there was a 71% decrease in specific [^{3}H]DHA binding with no effect of Gpp(NH)p on receptor loss. In addition, there was no change in the K_D value for [^{3}H]DHA binding to the remaining receptors after Carbo-Br pretreatment as compared to the control (control, 0.85 nM; Carbo-Br pretreated, 0.74 nM; Carbo-Br plus Gpp(NH)p pretreated, 0.87 nM). Figure 4-4 showed the same experimental protocol using cardiac ventricular membranes. After pretreatment with Carbo-Br or Carbo-Br plus Gpp(NH)p there was a 30 and 15% loss of specific [^{125}I]CYP binding sites, respectively. However, there was an increase in the K_D value for [^{125}I]CYP binding (control, 20 pM; Carbo-Br pretreated, 38 pM; Carbo-Br plus Gpp(NH)p pretreated, 38 pM).

Physiological effects of Carbo-Br. Figures 4-5 and 4-6 showed the ability of Iso and Carbo-Br to stimulate lung and heart ODC activity, respectively. Three hours after a 0.1 mg/kg injection of Iso there was an 8.0- and 8.6-fold increase in lung and heart ODC activity, respectively. Increasing the dose of Iso to 5 mg/kg increased the fold stimulation to 21- for both tissues. At a dose of 0.1 mg/kg of Carbo-Br in both tissues, there was an 8-fold stimulation of enzyme activity that was increased to 12-fold 3 hr after a 5 mg/kg dose. When propranolol (20 mg/kg) was given 30 min before the 5 mg/kg dose of either agonist, enzyme stimulation was completely blocked in both tissues. Finally, Figures 4-5

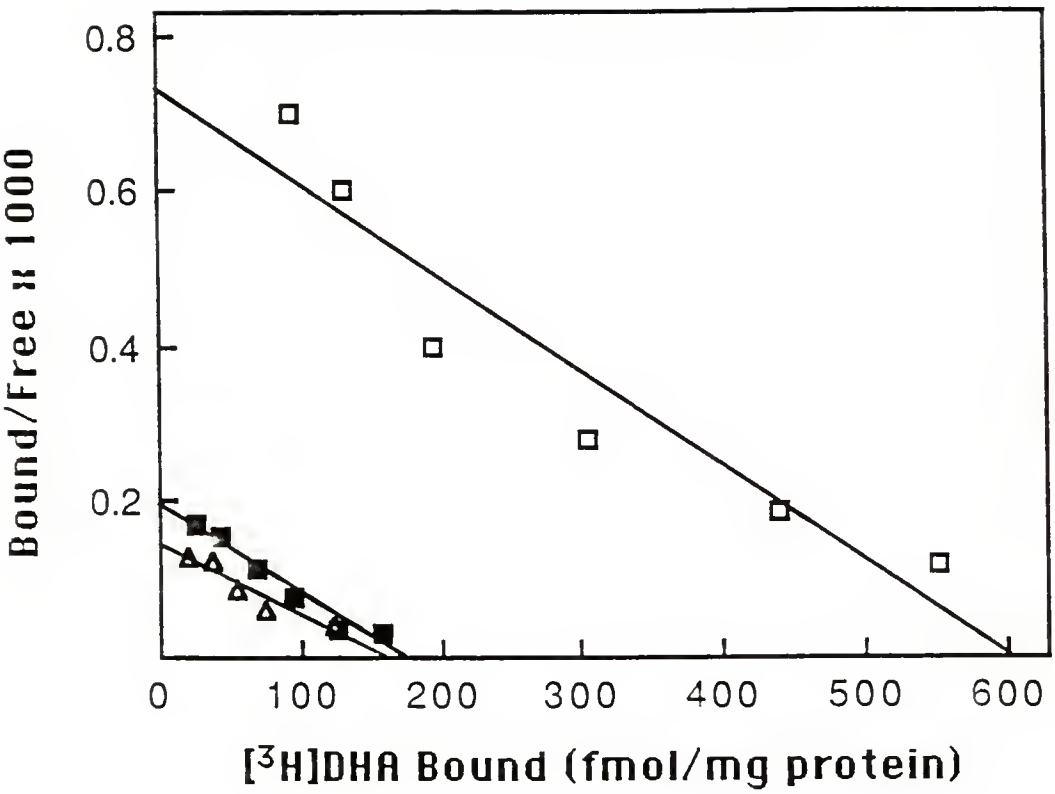

Figure 4-3. Scatchard plot of specific [^{3}H]DHA binding to rat lung membranes after treatment with Carbo-Br *in vitro*. Membranes were incubated without (open squares), with 0.5 µM Carbo-Br (closed squares), and with 0.5 µM Carbo-Br plus 100 µM Gpp(NH)p (triangles) for 30 min at 30°C. At the end of the incubation, the membranes were washed four times with buffer and assayed with 0.31-10 nM [^{3}H]DHA as described under "Methods". The data were plotted as the ratio of the amount of specifically bound ligand (pmol/mg protein) to free ligand (pmol/l) versus the amount of specifically bound ligand/mg protein. Data points were the mean of triplicate determinations and were representative of three experiments.

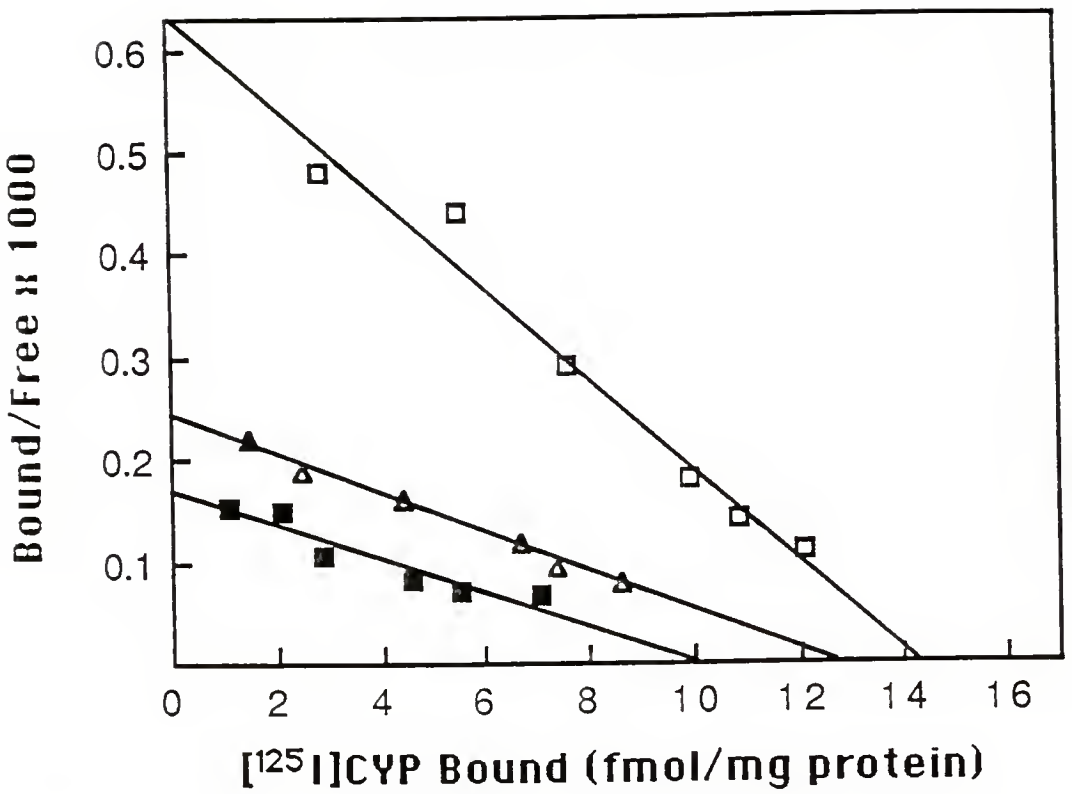

Figure 4-4. Scatchard plot of specific [^{125}I]CYP binding to rat heart membranes after treatment with Carbo-Br *in vitro*. Membranes were incubated without (open squares), with 0.5 µM Carbo-Br (closed squares), and with 0.5 µM Carbo-Br plus 100 µM Gpp(NH)p (triangles) for 30 min at 30°C. At the end of the incubation, membranes were washed four times with buffer and assayed with 3 to 100 pM [^{125}I]CYP as described under "Methods". The data were plotted as the ratio of the amount of specifically bound ligand (fmol/mg protein) to free ligand (fmol/l) versus the amount of specifically bound ligand/mg protein. Data points were the mean of triplicate determinations and were representative of three experiments.

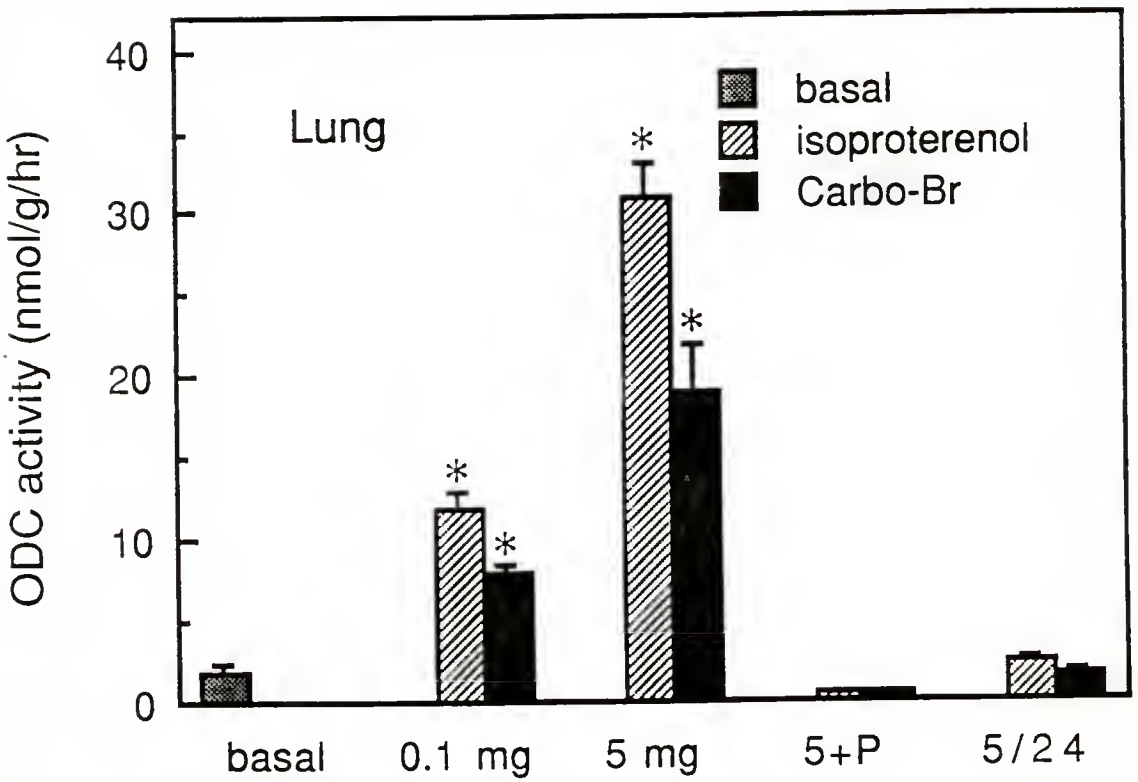

Figure 4-5. Stimulation of ornithine decarboxylase activity in rat lung 3 and 24 hr after *in vivo* Iso and Carbo-Br treatment. Male rats were injected i.p. with 0.1 or 5 mg/kg Iso or Carbo-Br, 20 mg/kg propranolol followed by 5 mg/kg Iso or Carbo-Br, or with vehicle alone. After 3 or 24 hr, lungs were removed and assayed for ODC activity as described in "Methods". Each response was the mean of three determinations assayed in triplicate (significantly different from basal levels, $^{*}p \leq 0.0005$).

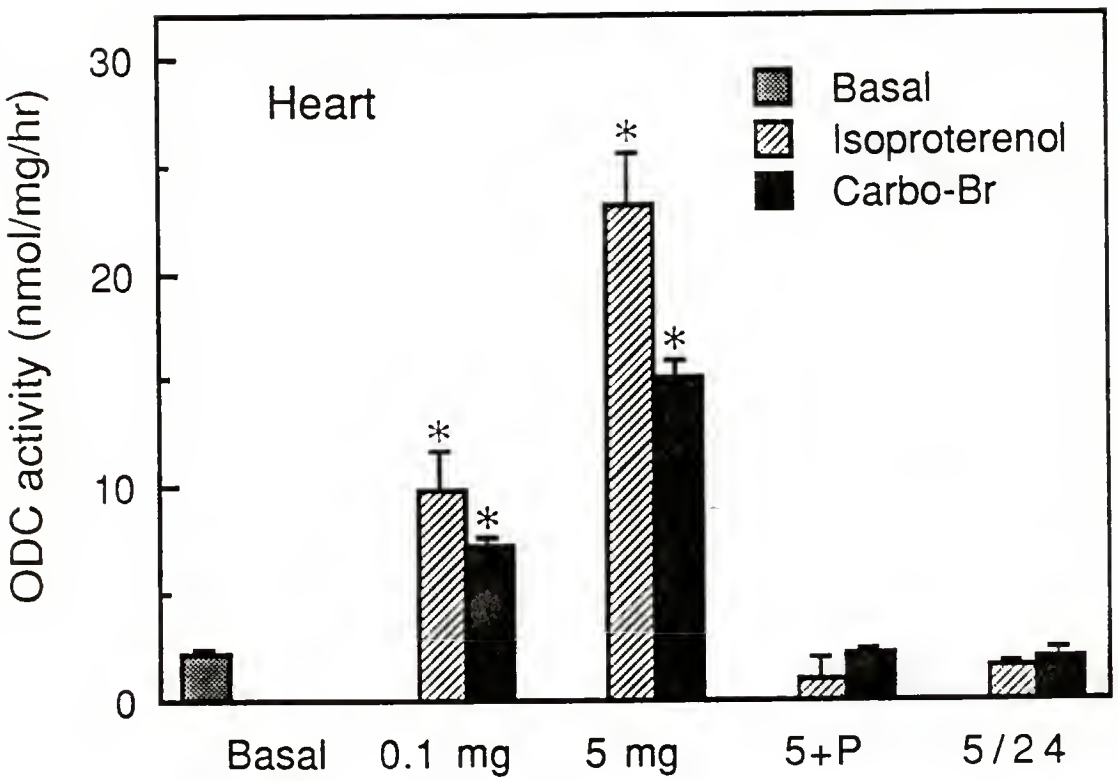

Figure 4-6. Stimulation of ornithine decarboxylase activity in rat heart 3 and 24 hr after *in vivo* Iso and Carbo-Br treatment. Male rats were injected i.p. with 0.1 or 5 mg/kg Iso or Carbo-Br, 20 mg/kg propranolol followed by 5 mg/kg Iso or Carbo-Br, or with vehicle alone. After 3 or 24 hr, hearts were removed and assayed for ODC activity as described in "Methods". Each response was the mean of three determinations assayed in triplicate (significantly different from basal levels, $^{*}p \leq 0.0005$).

and 4-6 showed that enzyme activity had returned to basal levels 24 hr after the 5 mg/kg dose.

A cumulative dose-response curve of Iso and Carbo-Br on sinoatrial beating rate was shown in Figure 4-7. The two curves were similar, with an EC_{50} value of 9.0 nM, indicating that Carbo-Br was a full agonist at the $beta_1$-adrenoreceptor. Figure 4-8 showed a time course of the stimulation of beating rate induced by 0.1 µM Carbo-Br and Iso. After 5 min in the presence of either agonist, there was an increase of 90-120 beats/min. When 50 nM nadolol was superfused the beating rate was reduced to slightly below the original resting rate. Addition of nadolol simultaneously with Carbo-Br resulted in little or no change from basal rates.

Table 4-2 presented the loss of specific [^{125}I]CYP binding sites in isolated right atria after a 20 min incubation with 0.1 µM Carbo-Br under conditions similar to those mentioned above. After a 20 min pretreatment with Carbo-Br, there was a 28% loss of binding sites in membranes from the right atria.

Carbo-Br effects on NE uptake. Rat cortical synaptosomes were prepared as described in "Methods". Pretreatment of these synaptosomes with various concentrations of Carbo-Br (10.0 nM - 0.1 mM) for 30 min prior to the addition of [^{3}H]NE resulted in an IC_{50} value of 20.5 µM for Carbo-Br inhibition of [^{3}H]NE uptake.

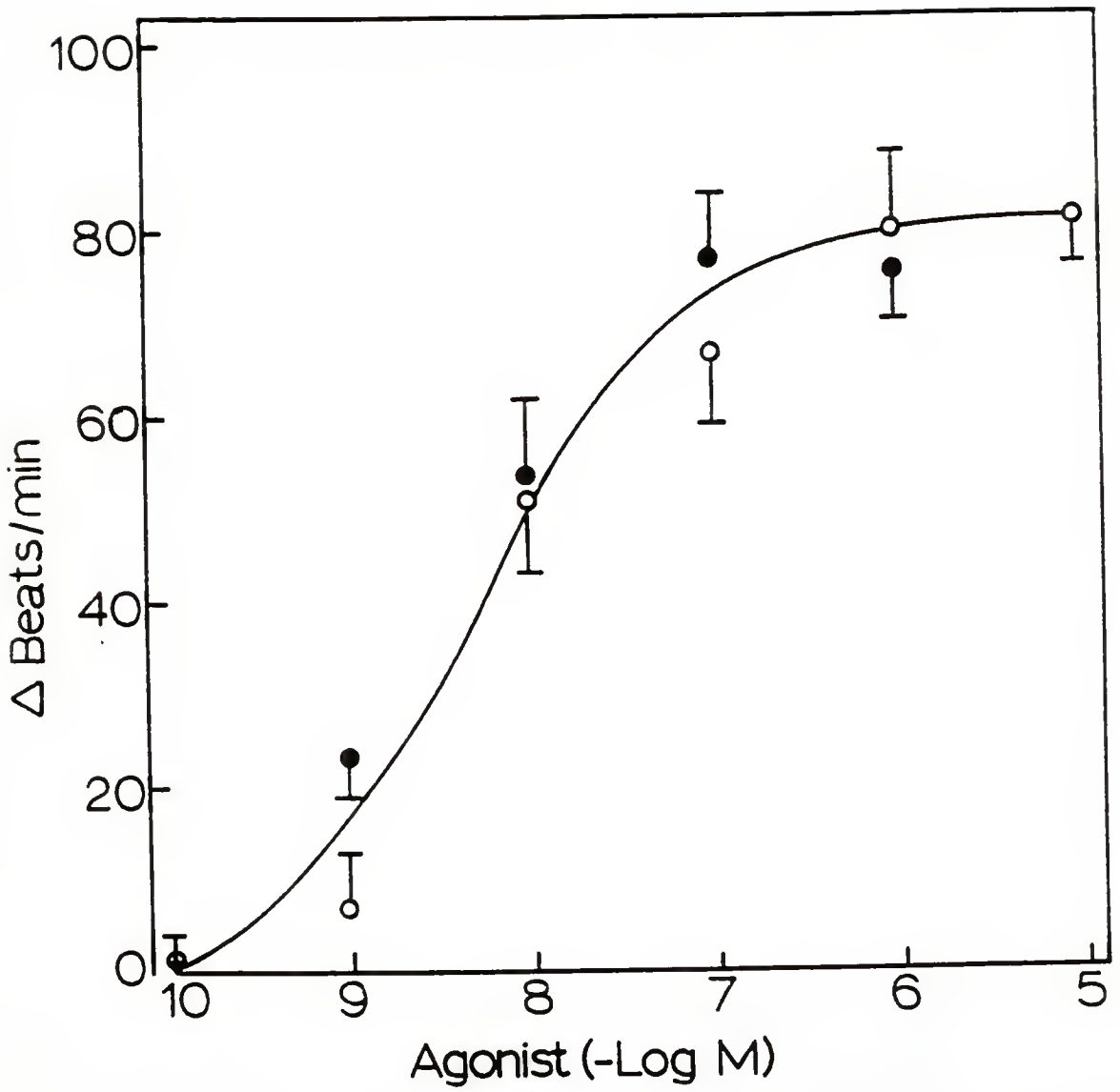

Figure 4-7. Stimulation of sinoatrial beating rate with Iso and Carbo-Br. The sinoatrial preparation was superfused with modified Tyrode's solution containing the indicated concentrations of Iso (closed circles) or Carbo-Br (open circles). Drugs were added for 5 min periods, starting with the lowest concentration, at which time the beating rates were recorded. Each point was the mean of four determinations.

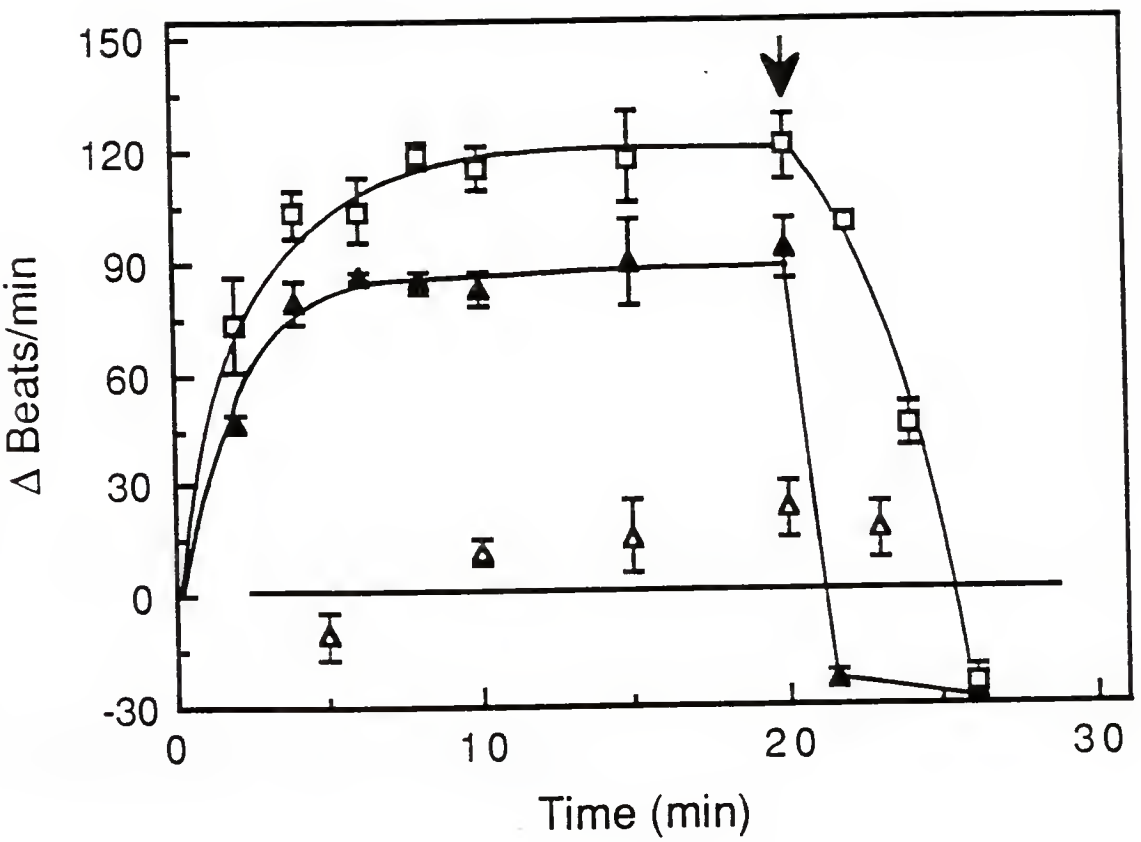

Figure 4-8. Time course of stimulation of sinoatrial beating rate with Iso and Carbo-Br. The sinoatrial preparation was superfused with modified Tyrode's solution containing 0.1 μM Iso (closed triangles), Carbo-Br (open squares) or Carbo-Br plus nadolol (open triangles) for 20 min, with beating rates recorded every 3 min. After 20 min, 50 nM nadolol was added to the superfusate and the beating rate recorded as before. Each point was the mean of three to four determinations. Beating rate with nadolol plus Carbo-Br was 14 beat/min.

Table 4-2. Loss of binding sites in right atria after Carbo-Br pretreatment

	B_{max} (fmol/mg protein)
Control	20.7 ± 0.7
Carbo-Br	14.9 ± 1.4[a]

Atria were incubated for 20 min at 36°C in HEPES/Tyrode buffer at pH 7.4 (bubbled continuously with 100% O_2) in the presence or absence of 0.1 μM Carbo-Br. At the end of that time, atrial membranes were prepared and washed 4 times. Membranes (75 μg/tube) were assayed with 3-100 pM [^{125}I]CYP as described in "Methods".

All values given as mean ± S.E., n = 5.

[a]Significantly different from control values, $p < 0.01$, as determined by paired t-test.

Discussion

The data from these studies indicated that Carbo-Br was a potent beta-adrenergic ligand *in vivo*. Carbo-Br was more potent than Iso at inhibiting specific binding to the receptor in all the tissues studied. In membranes from tissues where the same radioligand was used ([^{125}I]CYP), Carbo-Br was a more potent inhibitor (2-3-fold) in the spleen as compared to the heart and submaxillary gland. Since the spleen mainly contains beta$_2$-adrenoreceptors and the heart and submaxillary gland contain primarily beta$_1$-, this suggested a slight selectivity for the beta$_2$-receptor.

Quasi-irreversible or covalent binding after an i.p. or s.c. injection of Carbo-Br was indicated by the loss of specific [^{3}H]DHA binding sites in lung membranes, and [^{125}I]CYP binding sites in spleen, heart, and submaxillary glands, without a change in the K_D value for either ligand. Receptor concentration in both the lung and spleen decreased as a function of Carbo-Br dose. Indeed, the greatest loss of binding sites (70-75%) was seen in the lung and spleen, whereas the heart and submaxillary gland showed only a 15-25% loss of binding sites. Competitive binding studies with the beta$_1$-selective antagonist CGP-20712A in membranes from control rats determined the following beta$_1$ to beta$_2$ ratio of each tissue: lung, 22/78; heart, 75/25; spleen, 17/83; submaxillary gland, 82/18. Others have previously reported similar results (Barnett *et al.*, 1978; Rugg *et al.*, 1978;

Minneman *et al.*, 1979; Nahorski *et al.*, 1979; Bylund *et al.*, 1981). In all cases, the number of receptors of the $beta_2$-adrenoreceptor subtype in each tissue was proportional to the percentage of specific ligand binding lost. In fact, the $beta_1$ to $beta_2$ ratio increased significantly in all tissues 3 hr after a single 5 mg/kg injection of Carbo-Br: lung, 60/40; heart, 90/10; spleen, 45/65; submaxillary gland, 95/5. If Carbo-Br had shown no selectivity for receptor loss, then the subtype ratio would not have changed from control values. The fact that it did change (and the direction of change) indicated that the majority of receptor loss was at the $beta_2$-adrenoreceptor. It is probably unlikely, for several reasons, that the differential receptor loss between the various tissues was due to a drug accessibility problem. First, the same amount of receptor loss was noted regardless of whether the injection was made s.c. or i.p. This would indicate that absorption was not a problem. Second, the receptor loss in all tissues reached maximal levels at 5 mg/kg and was not increased further by increasing the dose. Finally, to our knowledge the heart and submaxillary gland (tissues that showed the least receptor loss) have not been known to have specialized barriers to small molecules.

The data from the present studies suggested that Carbo-Br was a beta-agonist *in vivo*. Two lines of evidence supported this. First, Carbo-Br induced the activity of ODC in both the heart and lung, and second, it increased the isolated atrial beating rate. The observation that

beta-antagonists blocked both effects indicated that the actions of Carbo-Br were mediated through the beta-adrenoreceptor. Carbo-Br was equally efficacious and potent as Iso for increasing the beating rate of isolated atria. This suggested that Carbo-Br was a full agonist, at least in this tissue. In contrast to beating atria, Carbo-Br induced a smaller increase in ODC activity as compared to Iso, suggesting that it was a partial agonist. However, some care in the interpretation of these data was required. Virtually nothing was known about the pharmacokinetics of Carbo-Br. Induction of ODC activity by a beta-agonist *in vivo* would depend upon the level of receptor occupancy and degree of activation. This, in turn, would depend upon the absorption, distribution and metabolism and/or excretion of the drug. At the lower dose employed (0.1 mg/kg), it was possible that the pharmacokinetics of the compound were such that less stimulation of the receptor was obtained than with Iso (i.e. Carbo-Br was absorbed less and metabolized or excreted faster). With the higher dose used, however, pharmacokinetics were probably not a possible reason for the lower enzyme induction by Carbo-Br. Substantial receptor occupancy was detected by the large receptor loss at this dose in several tissues (including the lung). Another possibility for the lower enzyme induction by Carbo-Br may be related to the rate of absorption of the drug. Enzyme activity was measured 3 hr after injection, a time shown to produce a maximal induction for Iso (Miska *et al.*, 1984). If Carbo-Br

was absorbed faster or slower, then the peak activity might not have been measured. In addition, Carbo-Br might have induced a rapid desensitization effect which might have also blunted the induction. Other possible complications to the interpretation of the ODC data were related to the possibility of an active metabolite of Carbo-Br stimulating enzyme activity. In addition, part of the ODC induction in the heart could be attributed to a reflex sympathetic drive due to a Carbo-Br induced drop in blood pressure. Nonetheless, the observation that Carbo-Br did stimulate ODC activity through a beta-adrenoreceptor supported the contention that it was a beta-agonist *in vivo*.

Even though beta-adrenoreceptors of both subtypes were present in the right atria ($beta_1$ to $beta_2$ ratio is 67/33), chronotropic and inotropic responses there were due entirely to stimulation of the $beta_1$-adrenoreceptor (Juberg *et al.*, 1985; Bassani & De Moreas, 1988). Our data were in agreement with these results as we noted a 28% loss of specific [^{125}I]CYP binding after a 20 min perfusion with Carbo-Br. We also noted a reversal of the chronotropic response by nadolol after a 20 min Carbo-Br perfusion. These results indicated that Carbo-Br affected both beta-receptor populations differently. The data indicated that Carbo-Br was equally potent and efficacious as Iso at stimulating the beating rate (EC_{50} = 9.0 nM). The drug bound competitively to one population of receptors ($beta_1$), since the stimulation <u>was</u> reversed. This implied that it was <u>another</u> population of

receptors ($beta_2$) that were binding Carbo-Br in a noncompetitive manner.

The question of whether Carbo-Br produced a sustained response *in vivo* has remained open but might depend upon the tissue or response under investigation. The data from the binding and spontaneously beating atrial studies would suggest that like Iso, Carbo-Br was a reversible $beta_1$-adrenoreceptor agonist. Therefore, the response duration would mainly depend upon agonist metabolism and/or elimination. In contrast, Carbo-Br bound extremely tightly and/or irreversibly to the $beta_2$-adrenoreceptor *in vivo*. Although the ODC data showed a loss of Carbo-Br induced activity at 24 hr, suggesting a desensitization effect, it should be pointed out that the reduction in ODC activity could be attributed to other mechanisms. For example, physiological reflex activities or increased rates of enzyme degradation might have been induced by Carbo-Br to blunt the possible long term effect. Therefore further studies are needed to establish if Carbo-Br could produce a long-term response or desensitization *in vivo*.

Recently, it was shown that BAAN, a partial beta-adrenoreceptor agonist which bound irreversibly to the receptor under certain conditions (Baker et al., 1985) could also bind irreversibly to the high affinity, sodium-dependent, norepinephrine uptake system (Baker et al., 1988). To determine whether Carbo-Br affected [^{3}H]NE uptake, we pretreated synaptosomes with Carbo-Br. Carbo-Br had an apparent IC_{50} value of

20.5 μM at inhibiting [^{3}H]NE uptake after pretreatment. This was 5-fold less potent than BAAN at inhibiting uptake, and several orders of magnitude less potent than its affinity at beta-adrenoreceptors.

CHAPTER 5
SUMMARY AND CONCLUSIONS

The present studies have shown that Carbo-Br was a potent, stable, full beta-adrenergic agonist. In the $beta_2$-adrenergic systems examined, Carbo-Br was 10 to 100-fold more potent than the racemically pure (-)isoproterenol. Since the Carbo-Br used in the present studies was a racemic mixture, the pure (-)isomer might be even more potent. In the $beta_1$-adrenergic system Carbo-Br may have been slightly more, or at least equipotent to, (-)isoproterenol. In both subtype systems, Carbo-Br was a full agonist. Both Carbo-Br and Carbo-Am produced sustaineded activation of adenylate cyclase activity in isolated membranes. The tight binding of Carbo-Am to the beta-adrenoreceptor appeared to be noncovalent whereas the quasi-irreversible binding of Carbo-Br might involve receptor alkylation. Carbo-Br bound extremely tightly to the beta-adrenoreceptors, and caused a desensitization of the beta-adrenergic system in intact cells. *In vivo,* Carbo-Br appeared to bind in a quasi-irreversible fashion to the $beta_2$-adrenoreceptor, while exhibiting full agonist properties at both receptor subtypes. The spectrum of binding and responsiveness characteristics of these carbostyril congeners were novel and worthy of much further study. Furthermore, other derivatives might be possible to refine to

direct these compounds towards more specific receptor actions.

The importance of an irreversible and/or radiolabelled beta-adrenoreceptor agonist could not be overstated. Some of the possible questions that could be answered by an irreversible agonist have already been discussed in Chapter three. Recent work by Dohlman and associates (1988) stressed the importance of a beta-adrenoreceptor ligand that would stay covalently bound to the receptor. Their attempt to locate and sequence the site of covalent incorporation of the beta-adrenergic antagonist [^{125}I]para-azidobenzylcarazolol into the beta$_2$-adrenoreceptor was only partially successful as the compound was subject to nonspecific cleavage during the initial cycles of protein sequencing. It seems an exciting possibility that a radiolabelled form of Carbo-Br might be used to identify and sequence the agonist binding site of the beta$_2$-adrenoreceptor.

In addition to the potential of Carbo-Br as an irreversible agonist, Carbo-Br and Carbo-Am might also prove to be highly useful as radiolabelled agonists. Because of the many differences between agonist and antagonist interaction with the beta-adrenoreceptor (discussed previously), there has been for measurement of direct agonist binding. Direct agonist assays would be potentially more sensitive than indirect competition studies using radiolabelled antagonists.

In the past, the agonists [^{3}H]hydroxybenzylisoproterenol ([^{3}H]HBI) and [^{3}H]epinephrine ([^{3}H]Epi) have been used with

limited success to directly study agonist-receptor interactions. Problems with [^{3}H]HBI and [^{3}H]Epi could be attributed to their low specific activity, extreme instability due to oxidation, and high nonspecific binding. In addition, [^{3}H]Epi only bound to high affinity sites. It could not be used to measure total receptor number, only the number of receptors that were able to form a ternary complex (i.e. it would not measure receptors in the low affinity conformation). The fact that Carbo-Br and Carbo-Am might be iodinated insures them of a high specific activity; furthermore, they were stable to oxidation. Of course, it is not known how much nonspecific binding these compounds would exhibit.

Since the beta-adrenergic system has played such an important role in health and disease, any compound that has an effect on the system has the potential for clinical drug development. As tight and/or irreversibly binding, beta$_2$-adrenoreceptor specific agonists which produce sustained receptor activation, one or more of these carbostyril congeners might be used to someday successfully treat asthma and other pulmonary disorders, glaucoma, premature labor contractions, and sudden infant death syndrome.

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BIOGRAPHICAL SKETCH

Kelly Standifer was born on April 5, 1962, in the charming little hamlet of Norman, nestled in the middle of the rolling plains of Oklahoma. She attended primary and secondary school there, graduating from Norman High School in 1980. Boldly, she left home to attend St. Gregory's College, a whole 90 miles distant. St. Greg's (as it was called) was a wonderful retreat into learning, full of dedicated and endearing monks, and filled Kelly with the confidence that she could tackle anything. After graduating from St. Greg's in 1982 with an Associate in Natural Science degree, Kelly left the friendly confines of Oklahoma for the undiscovered treasures awaiting her at Duke University in North Carolina. Assuming, quite incorrectly, that Duke was simply a progression to upper level coursework, and that the first priority was of a social nature, Kelly very nearly fled home after the first semester. Fortunately, she recovered her resolve and zest for learning, and actually graduated in May 1984 with a Bachelor of Science degree. It was at Duke that Kelly got her first glimpse of biomedical research. Convinced she had found her niche in life, Kelly applied to, and was finally accepted into, the graduate program in

pharmacology at the University of Florida in Gainesville. There, she found herself working under the tutelage of Steve Baker. Life in Steve's laboratory was never boring, and Gainesville was pleasant enough, but every year about basketball season Kelly longed for the classy style of basketball to which she had become accustomed at Duke. Despite these seasonal longings, Kelly finished her tenure at U.F., receiving her Ph.D. in August, 1988. Her next, and perhaps greatest, challenge awaits her in New York City where she has a postdoctoral fellowship with Dr. Gavril Pasternak at Memorial Sloan-Kettering Cancer Institute.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Associate Professor of Pharmacology
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Assistant Professor of Pharmacology
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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